AFFECTOR ELECTROBIOTECHNOLOGY

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ABSTRACT

In this thesis, aspects of mainly electric field effects on cellular systems are investigated, although electromagnetic effects on biological systems in general are also considered. The standard biotechnology processes of electroporation and electrofusion are shown to be electrically sub-optimal. Conventional d.c. pulses generate undesirable conditions such as asymmetrical membrane breakdown and cellular rotation. Normal electrical protocols also contribute to lower efficiencies by being sensitive to cell radii and other biological and physical variables.

Both physical and numerical models indicated that replacing d.c. pulses with a.c. pulses symmetrical about a zero potential axis, not only reduces the inherent problems of asymmetrical breakdown and cellular rotation, but also provides the means to reduce efficiency sensitivity to cell radii. This is important in fields such as human monoclonal antibody research and the generation of transgenic animals. In addition, improvements to existing d.c. multiple pulse systems can be made by using shorter time constants and reducing the magnitude of successive pulse electric fields.

The lysing effect of high magnitude electric fields are used in the study of disinfecting biologically contaminated liquids. It was earlier found that water could successfully be disinfected of the bacterial organism serratia marcescens. However, for higher liquid conductivities, applied electric field frequencies had to be increased so that electrolysis effects could be controlled. There is an upper limit to the frequency that can be used due to the cell membrane capacitance, which correspondingly puts an upper limit on the conductivity of the liquids that can be treated. Dead band regions where the applied a.c. electric field does not induce membrane dielectric breakdown, must be minimised. This suggests that a square wave should be used. Lower conductivity liquids are shown to provide the best opportunities for practical applications.

Cancer cell growth modification unique to the combined effects of low magnitude d.c. electric fields and silver ions was found to exist. The morphological changes suggest that possible dedifferentiation of the cells has been achieved. Other metal ions tested did not produce the same results. Silver ion effects also provided evidence of disinfection properties that can be utilised in some water treatment applications.

Resonant electromagnetic energy transfer into biological systems is investigated at a theoretical level. The resonant characteristic should enable specific targeting of

effects which are related to the energy and power applied. Resonant characteristics of low level millimetre wave electromagnetic fields are suggested to be involved with cell to cell interactions. Thus, immunological function could be directly related to and altered by those fields. Higher energy effects are more likely to act by direct physical mechanisms and are therefore more likely to result in useful applications in the short term.

ACKNOWLEDGEMENTS

In this section it is expected that thanks will be offered to many people. Be that as it may, I would still be acknowledging what the following people have done for me even if it was not traditionally part of a thesis.

I am extremely grateful for the good fortune of securing Patrick Bodger (Pat) as my supervisor. Apart from the unerring professional guidance, Pat always proved to be inspirational whenever I felt that maybe I was not cut out for a Ph.D. or that the project was faltering. Furthermore I am very happy to be able to consider Pat as a valued friend.

Heaven knows what my wife Donna has put up with during my postgraduate years. She has endured periods of grumpiness, depression, anger, misfortune and a fair amount of financial drought, all usually with a smile. I seriously believe that if Donna had not been by my side, the chances of me accomplishing what I have, would have been severely reduced.

My parents brought me up believing that the only limits in my life would be put there by me, and have they ever paid for that (literally). I will always be indebted to them. Thanks also go to my sister who kept the proverbial wool out of my eyes with a skilful hand and a lethally sharp pair of scissors.

Breaking into the field of biology put me in great need of coaching, guidance and resources. The two people central to meeting those needs were Dr Mike Bodger (Pats big brother) of the Haematology Research Unit., Christchurch Public Hospital, and Dr Peter Elder of the Steroid Unit, Christchurch Public Hospital. Even though I undoubtedly tried their patience with incessant questions and bungling proficiency, they taught me well and made me feel like I might have a reasonable aptitude for this kind of research.

During the course of my project, I required a not insignificant amount of technical assistance. In this capacity and more I would like to thank Dave Baran whose canny ability with electronics and obvious interest in the project and other topics, allowed me to complete what I have and was a marvellous discussion partner in many areas. I really appreciated the help from Jac Woudberg, especially in making sure I did not have a close encounter of the high voltage kind. I am sure Peter Lambert will be pleased to see the last of the drawings I presented him with of nigh on impossible to build constructions

(which he somehow did anyway). Many thanks to Dermot Sallis for his multiple text book-like memory and willingness to share that knowledge. Other highly appreciated technical assistance came in the form of excellent electron microscope operation by Mike Flaws, Mike Cusdins' great wealth of practical experience and weekly thrashings at squash administered by Nick Smith.

Without the direct help of Darin Hutcheson and Peter Crellen on the dedifferentiation experimentation, Marc Palmer building the water disinfection power supply and Brandon Lancastle in the silver disinfection experimentation, my thesis would not be the document it is. I wish them well in their engineering futures.

I have seen quite a number of postgrads come and go during my studies and I got to know most of them. A few in particular stand out as they offered friendship, encouragement and help without any expectations. Members of this group are Ken Johnson, Dave Hawkins, Robert van Nobelen, Gary Ballantyne, Stu MacDonald, Dave Gilbert and Mike Livingstone. I will consider myself very lucky if I continue to meet people like this in my future years.

Finally I would acknowledge all others who had some input or influence on my work. All of it was valuable and I was thankful to receive it.

GLOSSARY

Terminology

Antibody	Globular protein produced by activated B cells with extremely specific binding sites used in specific immunological processes.
Antigen	Complementary molecule to antibody binding site and trigger for antibody production by activated B cells.
B cells	B lymphocytes.
Bacterial cells	Prokaryotic cells.
Biocompatible	Does not induce an immune response in the organism.
Carcinogenic	Cancer inducing.
Cell membrane	Phospho-lipid bilayer that surrounds the constituents of all cells.
Cell nucleus	Membrane bound region where genome is restricted to.
Cell tissue	Structurally connecting cells.
Cell viability	Physical condition of a cell in its ability to procreate. Viable: it can. Non-viable: it can not.
Culture	An isolated solution of growing cells.
Culture dish	A type of container where cells can be grown.
Cytoplasm	All constituents of a cell excluding the cell membrane and the genetic material.
Dedifferentiation	Reversal of differentiation characteristics.
Differential regulation	Specific control of the genes in the genome during differentiation.
Differentiation	Changing cellular characteristics to a more specialised state.
Disinfection	Killing of almost all biological contaminates.

Electropermeabilization	Process of electrical induction of membrane dielectric breakdown leading to a higher membrane permeability.
Eukaryote	Single or multi-celled organisms that have a distinct nucleus.
Gene	DNA sequence that codes for an entire protein.
Genome	The entire set of genes in an organism.
Hybridoma	A cell that is the product of the fusion of two dissimilar cells.
In-vitro	Experiments performed on biological cells in culture.
In-vivo	Experiments performed on whole biological systems.
Ion pumps	Transmembrane proteins that drive ions either into or out of the cell interior.
Maturity	Degree of cellular differentiation.
Membrane permeability	Transparency of membranes to material transport.
Metabolite	Molecule used in the metabolism function.
Mitosis	A form of cellular division and multiplication.
Monoclonal antibodies	Antibodies all with the same binding site.
Multipotent cells	These cells have undergone a degree of differentiation past the pluripotent stage but still may differentiate into one of a number of different cells.
Oncogene	A naturally occurring gene, that when activated, has the potential to cause the cell to become cancerous.
Organelles	Functional multi-component units in the cytoplasm.
Ovum	Female gamete (or sex cell).
Pathological	Disease forming or inducing.
Pluripotent cells	These cells belong to the ectoderm, mesoderm, and endoderm families and may differentiate into any one of their possible products.
Prokaryote	Single celled organisms that do not have a nucleus.
Restriction enzyme	DNA cutting molecule.
Sperm	Male gamete (or sex cell).

T cells	T lymphocytes.
Tissue regeneration	Reconstruction of lost or damaged tissue to a morphologically correct level.
Totipotent cells	These cells are able to produce an entire organism if placed in an appropriate environment (have undergone little or no differentiation).
Transgenic	An organism that has had foreign DNA incorporated into its genome that is passed on to its progeny.
Virus	Metabolically inactive self contained units of genetic material that invade cells.
Yeast cell	Single celled type of fungi.
Zygote	Totipotent cell formed by the fusion of an ovum and sperm cell.

Abbreviations

α	Alpha dielectric dispersion.
AIDS	Auto-Immune Disease Syndrome.
ASA	American Standards Association.
β	Beta dielectric dispersion.
DMEM	Dubecco's Modified Eagles Medium.
DNA	Deoxyribonucleic Acid.
D.T.	Decay Time.
EM	Electromagnetic.
ESR	Electron Spin Resonance.
EXAFS	Extended X-ray Absorption Fine Structure.
FCS	Fetal Calf Serum.
γ	Gamma dielectric dispersion.
HIV	Human Immunodeficiency Virus.
H.M.E.F.	High Magnitude Electric Field.
IR	Infra Red.
MRI	Magnetic Resonance Imaging.
mRNA	Messenger Ribonucleic Acid.
NMR	Nuclear Magnetic Resonance.
Q1	Source current transistor.
Q2	Mirror current transistor.
RNA	Ribonucleic Acid.
RPMI	Roswall Park Memorial Institute.
UV	Ultra-Violet.
XANES	X-ray Absorption Near Edge Structure.

Mathematical Notation

a (cm)	Cell or balloon model radius.
C_m (F/cm^2)	Specific membrane capacitance.
d (cm)	Membrane thickness.
$\Delta U_m(t)$ (V)	Induced change in membrane potential at time t.
$\Delta\Xi(t)$	Induced change in membrane structure at time t.
E (eV)	Energy.
E_0 (V/cm)	A.C. applied electric field amplitude.
E_p (V/cm)	Constant electric field magnitude or capacitive discharge peak electric field magnitude.
$E(t)$ (V/cm)	Excitation electric field at time t.
$E(\theta)$ (V/cm)	Excitation electric field at time θ.
ϵ_0 (C^2/(N m^2))	Permittivity of free space.
ϵ_r	Relative permittivity.
$F(\sigma)$	Conductivity factor.
h (J s)	Plank's constant.
$M(t)$ (V)	Membrane dipole moment.
ω (radians/s)	Angular frequency.
ϕ (radians)	Angle from a point on the membrane surface to the axis which is parallel to the applied electric field and passes through the origin of the cell or balloon model.
R2 (Ω)	Current setting resistor.
R_m (Ωcm^2)	Specific membrane resistance.
ρ_e (Ωcm)	External suspension medium resistivity.
ρ_i (Ωcm)	Internal resistivity of cell or balloon model.
σ (S/cm)	Variable of conductivity.
σ_e (S/cm)	External suspension medium conductivity.
σ_i (S/cm)	Internal conductivity of cell or balloon model.
σ_m (S/cm)	Membrane conductivity.
T_r (s)	Membrane relaxation time constant.
t (s)	Variable of time.
θ (s)	Time variable of intergration.
U_{m0} (V)	Induced a.c. transmembrane potential amplitude.
$U_m(t)$ (V)	Membrane potential at time t.

PREFACE

For a number of years, electric and magnetic fields have been major components of many processes in the medical arena. To name all of the processes here would be impractical if not impossible. Be that as it may, it is worth mentioning some of the more widely used and important techniques. The electrocardiogram (ECG or EKG), and electroencephalogram (EEG), have provided essential information on the functioning of the heart and brain [Cromwell *et al.* 1980]. Nuclear magnetic resonance (NMR) [Hollas 1982], magnetic resonance imaging (MRI) [Morris 1986] and X-rays [Lange *et al.* 1989] have resulted in the ability to determine molecular structures and to non-invasively obtain detailed visual information on internal bodily objects. There is a common factor in these named processes. They are all diagnostic tools. This is by far the prevalent application of electromagnetics in medicine. Use of electromagnetics to affect biological systems is considerably less common.

Prior to the 20th century, many electrotherapeutic techniques existed [Becker 1990]. However, modern science indicated that most of these techniques were without any observable scientific foundation. A dogma resulted and any use of electromagnetics in therapies or to affect biological systems, was frowned upon. It was not until after World War Two that the scientific community began to relax its position. Advancements in microwave generation resulted in the observation that soft tissue could be uniformly heated by its application [Field and Franconi 1987]. Exposure of living tissue to γ-rays indicated that there was a favourable killing rate differential between normal cells and cancer cells [Swan 1981, Mansfield 1983]. Laser technology was found to be useful in a number of applications from eye treatments to cauterising scalpels [Fuller 1987]. In virtually all cases where electric, magnetic or electromagnetic (em) effects could be used in a medical application, significant improvements over the practiced processes was achieved. In spite of this trend, very little research has been carried out in biology and medicine on specifically trying to find practical effects of electromagnetics. This is opposed to the substantial amount of work that has been and continues to be carried out on the causal effects of electromagnetics in biological systems. This research does not actively look for applications but has resulted in the applications mentioned above and others. Some of these applications are components of this thesis.

Chapter 1 is the only chapter in this thesis that does not contain any original work.

Its sole purpose is to provide brief background information on biology for those readers not conversant in that field.

Discovered high magnitude electric field effects on cellular membranes has directly led to three different applications. One known as electroporation is investigated in Chapter 2. Electroporation utilises the high cellular membrane permeability (or porosity) state that is induced through the application of high magnitude electric fields, to selectively transport materials of interest across the membrane. A physical 'balloon' model of a cell is introduced and yields results that were previously unobservable. Modifications to the electroporation technique are then suggested.

Cells permeabilised similarly to electroporation that are brought into close contact, tend to fuse and form hybridomas. This effect is used in a process called electrofusion and is studied in Chapter 3. It is shown through numerical and physical modelling that a.c. signals symmetrical about a zero potential axis should result in more efficient electrofusion and electroporation. It is also indicated that using such waveforms should increase controllability of the techniques.

In both the electroporation and electrofusion processes, application of electric fields with excessively high parameters results in massive loss of cell viability. Chapter 4 looks at using this effect to disinfect biologically contaminated liquids. The major liquids concentrated on are water and hydrocarbons such as kerosene and diesel. The applicability of high magnitude electric field (h.m.e.f.) killing of liquid borne biological contaminates is shown to be mainly determined by the conductivity of the liquid which produces upper and lower bounds.

Perceived changes to cellular maturity and genetic expression by the application of very low magnitude d.c. electric fields in the presence of particular ionic compounds has indicated at possible applications in regeneration and cancer therapy. Initial investigations and experimentation in this vain are described in Chapter 5. Cancer cells of morphologically different backgrounds, exposed to low level d.c. electric fields and silver ions can be seen to convert to a common morphology. This is proposed as being an indicator of cellular dedifferentiation.

In contrast to probing already existing processes, Chapter 6 is effectively an exploration in the possibility of using resonant em energy transfer for various medical applications. More specifically in applications that require the ability to precisely target certain biological aspects. These are discussed with respect to different energy and power levels which directly determines the mechanisms of action.

The thesis as a whole is concluded in Chapter 7. The main results are briefly summarised and future work is proposed.

At the onset of this thesis in 1992, electroporation and electrofusion processes were going to be investigated to determine if any improvements in their electrical aspects could be achieved. This was accomplished. However, owing to a high degree

of explorative freedom, other avenues were travelled along. This is the main reason for the relatively diverse range of chapter topics. It is my belief that the result has provided me with a broader understanding in the field that interests me. Engineering philosophy encourages such understanding as it is the ability to bring together diverse ideas to form a functional and novel creation, that forms the foundation of a good engineer.

All of the biological experiments performed as a direct part of this thesis were co-ordinated and supervised by either Dr Michael Bodger of the Haematology Research Unit, or Dr Peter Elder of the Steroid Unit, Christchurch Public Hospital. Their expertise also ensured that results were not misconstrued and that unrealistic presumptions and expectations were pointed out.

All images in this thesis that originate from photographic sources, have been digitally scanned and contrasted. This was essentially done to make generation of multiple copies simple. Some of the figures in Chapter 2 and Chapter 3 had their contrast enhanced to highlight details that might otherwise be lost in reproduction.

Papers prepared during the course of this thesis are listed below in the approximate order of preparation.

Gaynor, P.T. and Bodger, P.S. 'Ionisation of dielectric spheroid membranes: A balloon model of electroporation of biological cells', *IEE Proceedings-A*, Vol. 141, No. 3, 1994, pp. 190-196.

Gaynor, P.T. and Bodger, P.S. 'Electrofusion processes: Theoretical evaluation of high electric field effects on cellular transmembrane potentials', *IEE Proceedings-A*, accepted for publication, May 1994.

Gaynor, P.T. and Bodger, P.S. 'A balloon model of biological cell electropermeabilization in relation to the radius dependence of membrane dielectric breakdown', *IEE Proceedings-A*, accepted for publication, November 1994.

Bodger, P.S., Gaynor, P.T., Hutchinson, D.G. and Crellin, P.J. 'In-vitro cell growth modification by combined d.c. and silver ion action', Submitted to *Biophysical Journal*, November 1994.

CONTENTS

Chapter 1

BACKGROUND

This chapter essentially acts as a basic terminology coach for those who have had little exposure to biological theory. Owing to the vast amount of even basic theory, only those topics relevant to the thesis will be backgrounded. The interested reader can find further comprehensive basic biology in the book by H. Curtis and N. S. Barnes, called 'Biology'. Each other chapter contains the relevant theory required independently of this one. Readers conversant in basic biology may, if they wish, pass over this chapter.

1.1 THE CELL

Most of the content of this thesis deals with electromagnetic characteristics of and effects on biological cells. More specifically, experimentation is conducted on some bacterial and mammalian cells. It is then pertinent to give a brief overview of cell physiology and dielectric properties.

There exists what is known as the 'cellular theory', which states that all organisms are composed of cells. This is one of the few fundamental statements in biology [Hoppe *et al.* 1983]. The cell is thermodynamically an open system which is in constant exchange with its environment. It is like a microscopic factory that takes in materials and uses its internal machinery to convert those materials into something else which may go into making new machinery, factory expansion and multiplication, power, exportation or defence from rival factories.

Cells are made up from a set of common components. Any particular cell type is determined by the combination and amount of those components. All cells have two components in common. These are the cell membrane and some form of genetic material [Curtis and Barnes 1989a].

In the biological sense, a cell membrane is a physical barrier between the functional cell constituents and the surrounding environment which allows the selective transfer of material essential to cell survival. The organisation of the cells genetic material basically determines two fundamental types of cell, the prokaryotes and eukaryotes.

Prokaryotes (meaning before a nucleus) concentrate most of their genetic material to a loosely defined region called the nucleoid near the cell centre. Eukaryotes (meaning true nucleus) separate most of their genetic material into a well defined region, called the nucleus, with a double membrane sack known as the nuclear envelope. All bacteria are prokaryotic and all animal and plant cells are eukaryotic.

In addition to a cell membrane, prokaryotic cells also have a cell wall which is produced by the cell and resides on the outer surface of the cell membrane. Some eukaryotic cells also produce cell walls but are quite different to those of the prokaryotes. Animal cells do not have cell walls.

The rest of the components of a cell are grouped as constituents of the cytoplasm. The cytoplasm contains all the molecules required for existence and other well defined regions of function known as organelles (meaning small organs).

All cells contain very small organelles called ribosomes. Ribosomes are the construction sites of protein synthesis. Prokaryotes can contain a few other organelles such as storage granules (of substances like glycogen), flagella, cilla, microfilaments, microtubules and centrioles. Flagella and cilla are evaginations of cell membrane bound microfilaments and are involved with motility. Microfilaments, microtubules and centrioles are all protein based physical construction materials involved with cell shapes, movement, division and internal structure. Eukaryotic cells contain most of these and many other more complex organelles that usually involve membrane material. The major organelles of this type are , endoplasmic reticuli, golgi complexes, lysosomes, peroxisomes, mitochondria, and in plant cells plastids and vacuoles. A sectional representation of an animal cell is shown in Figure 1.1 [Curtis and Barnes 1989a]. The endoplasmic reticulum is a labyrinth of membrane material and is divided into two forms, rough and smooth. Rough endoplasmic reticulum has attached ribosomes whereas smooth endoplasmic reticulum does not. Molecules synthesised here are put into membrane bound vesicles and transported to the golgi complexes.

Golgi complexes are constructed as loosely stacked sacs. They act as packaging and distribution centres for molecules produced in the cell. Large vesicles produced by the golgi complexes are lysosomes and peroxisomes.

Lysosomes contain hydrolytic enzymes that are used by the cell to break down imported proteins, polysaccharides and lipids. Peroxisomes contain lytic enzymes that break down purines and other similar compounds.

Mitochondria are large and very important membrane bound organelles that supply energy giving molecules for most cellular processes. The number of mitochondria in a cell is determined by the relative energy requirements of that cell. Mitochondria also contain their own genetic material.

Plastids are large organelles found in plant cells. They have three distinct forms, leucoplasts (where starch is often stored), chromoplasts (which contain orange and

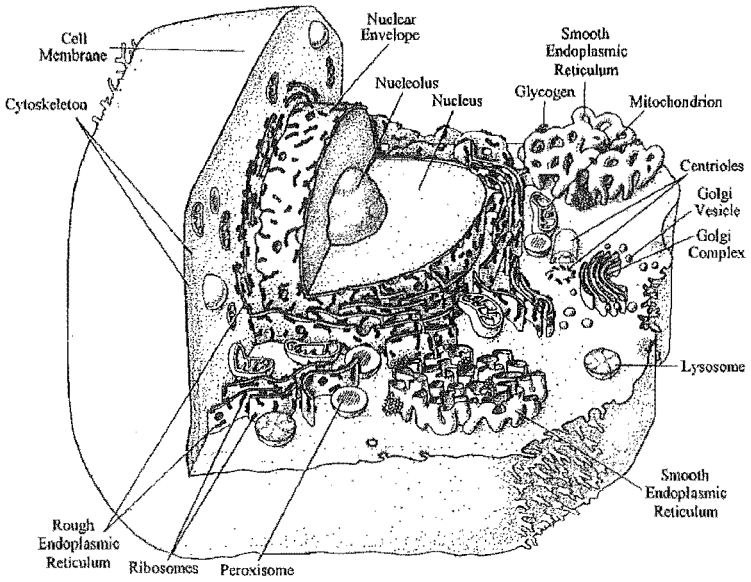


Figure 1.1 Sectional view of a typical animal cell and its components

yellow pigments) and chloroplasts (chlorophyl-containing plastids where photosynthesis takes place).

The way a cell uses its molecular make up and organelles to function is beyond the scope of this chapter. Suffice to say that cell functioning as a whole is exceptionally complicated and affecting any of the organelles significantly alters those functions.

1.1.1 The Cell Membrane

Much of the thesis is concerned with the high magnitude electric field effects on cell membranes. Therefore, the basic physical structure and dielectric properties of most cell membranes are introduced here.

The fundamental building block of cell membranes is the phospho-lipid. These consist of a hydrophillic phosphoric acid and a pair of hydrophobic fatty acid chains. In a cell membrane, these molecules are arranged in two sheets which form a bi-layer. The phospho-heads cover the two surfaces of the bi-layer and the fatty acid tails constitute the interior of the bi-layer. This arrangement is shown in Figure 1.2 [Brown 1982]. Also depicted in Figure 1.2 are globular proteins that reside in cell membranes. Most of these proteins are involved with selective molecule transport across the membrane or with molecular receptors. Proteins like these can constitute up to 60% of the cell membrane volume. It is the normal function of some of these proteins that produce inherent membrane potentials in cells.

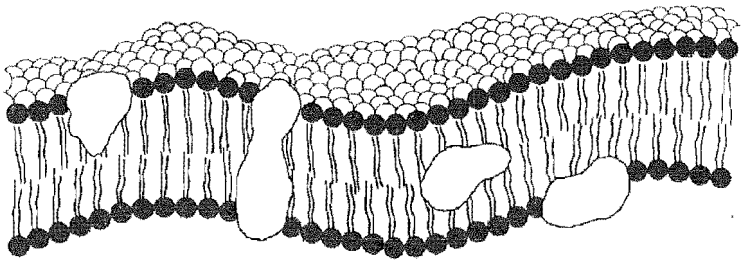


Figure 1.2 Fluid mosaic model representation of cell membrane material. The small spheres represent the lipid phospho-heads and the thin tails represent the fatty acid sections. Also shown are typical placements of membrane globular proteins.

The lipids and proteins that make up a cell membrane are not fixed in their structural positions. They can readily move transversely to the plane of the membrane and also have the ability to rotate or flip across the membrane plane (although this is much less common). These motional characteristics have resulted in the formation of the 'fluid-mosaic' model of the cell membrane.

Comprehensive data has been produced on the passive dielectric properties of cellular membranes from about 1Hz to 100GHz [Pethig 1984, Schwan 1985, Pethig 1988]. As a simple approximation, the cell membrane can be dielectrically modelled by a capacitor in parallel with a high magnitude resistor. Thus, as frequency increases, the impedance of the membrane decreases. If impedances on either side of the membrane remain relatively unchanged over a particular frequency range, then potentials developed across the membrane by an applied electric field will decrease. This effect is characterised by what is known as a Beta (β) dispersion in the relative permittivity (ϵ_r) for a bulk suspension of a particular cell type. The change in ϵ_r of a standard colloidal cell suspension is shown in Figure 1.3 [Schwan 1985]. There is also an alpha (α) dispersion due to the inherent transmembrane potential polarisation of associated molecules and a gamma (γ) dispersion due to the relaxation of free water in the suspension. Most of the high magnitude electric field investigations deal with relative permittivities between the α and β dispersions. More detailed information on the passive and dynamic dielectric behaviour of cell membranes is given in Chapter 2 and Chapter 3.

1.1.2 Multicellular Organisms

Whenever it takes more than one cell or cell type to constitute an organism, then these organisms are termed as multicellular [Curtis and Barnes 1989a]. A person is a multicellular organism in which over 200 specialised cell types exist. When cells of a particular type are physically grouped together, they are known as cell tissue. Thus people have muscle, skin and bone tissue. Organisation of this tissue represents an extraordinary level of complexity.

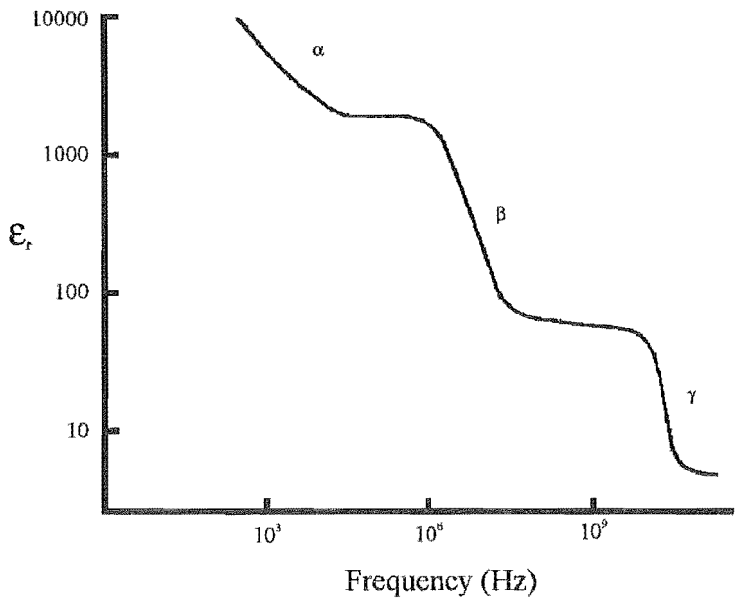


Figure 1.3 Relative permittivity dispersion curve for a colloidal suspension of E-coli cells. The main dispersion sites are labelled.

The behaviour of a single celled organism in an environment is quite different to the behaviour of a multicellular organism in the same environment. Interactions between cells can invoke responses to stimuli that have no effect on unicellular systems. In this manner, electromagnetic characteristics of effects on multicellular systems can be very different to those of unicellular systems.

1.2 DNA AND GENETICS

In Section 1.1 it was stated that all cells have some sort of genetic material. Genetic material is responsible for all the physical aspects of an entire organism. In the most basic terms, genetic material is a particular type of molecule that contains biological information in code. The most common form of this molecule is deoxyribonucleic acid (DNA) [Hoppe *et al.* 1983].

1.2.1 DNA

In basic terms DNA is an exceptionally long molecule which is essentially a tape of coded information. It has the form of a double helix (of alternating units of deoxyribose and phosphate) laddered by complementary coupled pyrimidines and purines [Brown 1982]. A representation of this form is shown in Figure 1.4. In total, two pyrimidines and two purines code in sets of three for a particular amino acid. There are only 20 amino acids that are used to make up all biologically synthesised proteins.

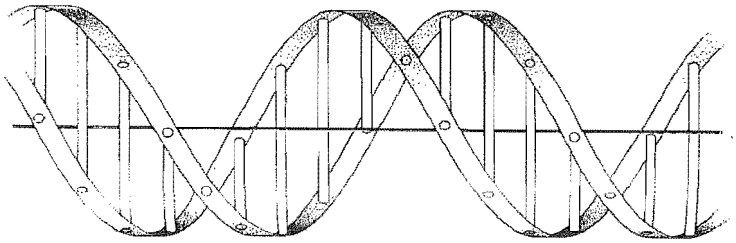


Figure 1.4 Simple structural representation of the DNA molecule.

1.2.2 Genetics

When the code in DNA is to be expressed, the double helix is parted between the purine and pyrimidine bonds and a strand of ribonucleic acid (RNA) is produced. RNA is much like DNA except it is single stranded. Deoxyribose is replaced by ribose and one of the pyrimidine types is replaced by another. The RNA is then 'read' by a ribosome which attaches amino acids together in the order stated to produce proteins.

The DNA sequence that codes for a complete protein is known as a gene. It is therefore genes or combinations of genes that characterise the entire physical makeup of an organism in a particular environment. Genes determine the colour of eyes, the size of limbs and susceptibility to many ailments.

When cells divide, all of the DNA in the parent cell is copied (or replicated) so that both daughter cells have a complete copy of that DNA. As in all information storage systems, errors are made during replication. Inherent 'proof reading' and 'repair' molecules correct most of these errors. However, if there are too many breaks or errors in the DNA, some will not be intercepted. This can lead to changes in genes. The result may be a mutation in some physical characteristic [Curtis and Barnes 1989a]. Mutations are most likely to be detrimental to an organism and may result in the loss of viability. In rare instances, mutations can be beneficial if the altered physical aspect makes the organism more competitive against other organisms in its environment.

Many external conditions can cause genetic errors. Some chemicals and radiation produce specific mutations that can kill cells or disrupt their growth mechanisms which may lead to some forms of cancer. Changes in genes can also be induced by some viral bodies.

Viruses are metabolically inactive self contained segments of genetic material. Their sole function is to replicate. They do not have the means to do this and therefore invade cells to use their replicating and expression components. Once the cell has replicated the genetic material and produced more viral containers, the viral bodies often break out of the cell to invade others. In the process of breaking out, the host

cell usually dies due to excessive membrane damage. Some viruses can incorporate their genetic material into the host cells' genetic material. This can lead to genetic mutations with the associated problems described above. Normally though, the genetic incorporation has no adverse effects and is a state of latency for the virus. At a later time, the viral DNA usually dissociates from the host cell DNA and once again becomes active.

It is possible to incorporate foreign, yet specific, genetic material into cells and literally fool them into expressing for that DNA. This is known as transformation or transfection. In this manner, bacterial cells have been induced to produce human insulin in large quantities. If the genetic transformation is maintained and carried on to the organism's progeny. The organism can then be labelled as transgenic [Larrick and Burck 1991]. Transgenic fish are being produced that have had foreign genes added to their genetic material [Inoue *et al.* 1990]. These genes express for attributes such as increased resistance to disease and size and are passed on to the fish offspring.

Every cell (bar some extremely specialised cells) in a multicellular organism carries a complete copy of the organism's genetic material (or genome). The genome contains all of the genes for the entire organism. Therefore, in order to have a muscle cell, all the genes that express for other cells must be 'turned off'. This is the case for all cell types and is known as differential regulation [Larrick and Burck 1991]. One of the common dogmas in biology essentially states that differential regulation can only switch genes off. That is, from sexual conception, cells can only become more specialised and specific in function. Recent studies have shown that under some conditions, genes can be turned back on, thus making the cell less specialised. The effect is called dedifferentiation.

Another way cells change their genetic characteristics is by fusing with other cells to form hybrids or hybridomas. Some different cell types with different characteristics can come into close contact and have their membranes fuse together to become one. The nuclei of the original cells fuse in a similar manner and lump their genomes together. The hybrid cell and its progeny then have the functional characteristics of the two original cells.

1.3 IMMUNOLOGY

In Chapter 3, a technique that has a substantial bearing on immunology research is presented and discussed. Basic immunological facts are introduced in the following sections. The mechanisms of immunological action are also addressed in Chapter 6.

Multicellular organisms have immune systems that guard against invasion by other organisms or foreign matter. In animals and especially mammals, the immune system is very complicated and sophisticated.

Immune responses in mammals can be roughly grouped into two types, non-specific and specific [Roitt *et al.* 1985].

1.3.1 Non-Specific Immunity

As a first line of defence against invasion by microorganisms or particles, a physical barrier exists in the skin and mucous membranes. If these initial barriers are breached, then an inflammatory response is engaged. Inflammatory responses are performed by general organism and particle engulfing white blood cells (phagocytic cells) known as granulocytes and monocytes. It is generally thought that these cells recognise the foreign bodies by their unusual chemical excrements. However, other stimuli may also be involved.

1.3.2 Specific Immunity

Organisms that evade the non-specific immunity system then encounter the specific immunity system. This system is based on the actions and interactions of two white blood cell types known as B lymphocytes and T lymphocytes (or commonly B cells and T cells).

B cells instigate what is known as an antibody response. Antibodies are globular proteins that have extremely specific binding sites. Specific antibodies bind to specific antigens (short for antibody-generating substance). Virtually all foreign proteins can act as antigens. For example, a bacterial cell is likely to have a number of different antigens on its surface.

B cells have on their surface the same type of binding site as antibodies. When a B cell comes into contact with an appropriate antigen, it turns into a plasma cell and starts producing large numbers of complementary antibodies for export. A purified population of one type of antibody is known as a monoclonal antibody population. When the antibodies bind to an antigen they act in one of three ways. They may coat the foreign bodies and make them clump together so that the inflammatory response can take over. The antibodies may combine with the antigen in such a way as to disrupt some vital activity in the foreign organism. Finally, the antibodies could attract naturally occurring lysogenic substances in the body.

Antibody response normally acts against viruses, bacteria and the toxins they may produce. Immune response against other eukaryotic cells is mediated through T cells. Three types of T cells are evident, cytotoxic, helper and suppressor T cells.

Cytotoxic T cells seek out foreign or infected self eukaryotic cells recognised through similar mechanisms as those involved with antigens. When found, the cytotoxic T cells release either phagocytic cell attractor chemicals, or cytotoxic chemicals that lyse the target cells directly.

Helper and suppressor T cells regulate the entire immune system. Helper T cells enhance both B cell and cytotoxic T cell function and proliferation. When the invader has been successfully eliminated, suppressor T cells quickly reduce the activity of the other immune cells.

The full interactions of cells in the immune system are still not understood. It is evident that other unknown stimuli and signals are involved in many aspects of immune responses.

Chapter 2

ELECTROPORATION

2.1 INTRODUCTION

During the formative years of modern micro and molecular biology, chemical and biological techniques were developed to transfer selected material through cellular membranes [Ausubel *et al.* 1990]. The ability to perform transmembrane transport of material is critical to many areas of biological research. Much of this research requires transport of macromolecules such as DNA, RNA or antibodies, chemical drugs, metabolites, molecular probes and various vesicles.

For any particular application, choosing a given transfer process is based on its efficacy, ease of use and side effects (if any). A characteristic most of the chemical and biological techniques share is that they are usually cell-type dependent and have relatively poor efficiencies. As such, methods which are versatile yet efficient are always being searched for and investigated. Electroporation is such a method.

The possibilities of using the dielectric breakdown of cellular membranes as a transport mechanism had been postulated in the early 1970's [Coster and Zimmermann 1975a, Zimmermann *et al.* 1973]. Research pertaining to membrane dielectric breakdown was carried out in the following years. The term electroporation was coined as a result of the observation that dielectric breakdown of cell membranes appeared to generate holes or pores that material could pass through [Hofmann and Evans 1986]. It has subsequently been shown that the idea of a hole is overly simplistic and that the dielectric breakdown induces a state of membrane permeability [Chang 1992]. Thus, the process is commonly called electroporation whereas the act of membrane dielectric breakdown is usually referred to as electropermeabilization.

Over the past ten or fifteen years the electroporation process has been used to affect material transport in an extremely wide range of prokaryotic and eukaryotic cells [Chang *et al.* 1992b, Neumann *et al.* 1989]. These uses include DNA and RNA transfection of bacterial, yeast, mammalian and plant cells. Uses also include introduction of molecules such as restriction enzymes [Morgan *et al.* 1990, Tsongalis *et al.* 1990], fluorescent and radioactive probes [Hashimoto *et al.* 1989] and antibodies [Chakrabarti

et al. 1989].

More recently, electroporation has been used in attempts to produce transgenic animals [Inoue *et al.* 1990] as an alternative to time consuming and extremely specialised microinjection techniques. Electroporation has been applied to in-vitro and in-vivo transient gene assays in animal and plant tissue [Dekeyser *et al.* 1990, Pu and Young 1990]. Receptor proteins important to AIDS research have been electroinjected into cell membranes [Zeira *et al.* 1991].

Electroporation has been investigated and refined to an extent that, for most applications, it is simpler and more efficient than rival chemical and biological processes [Chang *et al.* 1992b, Neumann *et al.* 1989]. However, the mechanisms of electropermeabilization are still not fully understood and there are aspects of the process which are, as yet, sub-optimal in their possible performance.

The scope of this chapter is to address some of these problems in an attempt to increase the understanding and efficiency of the electroporation process.

2.2 DIELECTRIC MEMBRANE RESPONSES TO HIGH MAGNITUDE ELECTRIC FIELDS

2.2.1 The Induced Transmembrane Potential

In order to achieve permeabilization of the membrane, an external electric field of relatively high magnitude (kV/cm range) is applied to colloidal cells via parallel electrode pairs. For most applications, biologically compatible suspension media is used which is assumed to be conductive relative to the membrane. Due to the nature of the system, the applied electric field induces a dipole across the membrane as shown in Figure 2.1. The dipole moment $M(t)$, at time t is calculated as [Holzapfel *et al.* 1982],

$$M(t) = \left(\frac{-2\pi\epsilon_0 a^3}{T_r}\right) \int_{-\infty}^{t} E(\theta) e^{\left(\frac{t-\theta}{T_r}\right)} d\theta \tag{2.1}$$

where ϵ_0 is the permittivity of free space, a is the cell radius, θ is the time variable of integration, $E(\theta)$ is the excitation electric field at time θ and T_r is the relaxation time constant of the membrane. The upper limit of the integral is the function variable of time. Thus, the integral must be evaluated individually for all t. The lower limit of the integral is set to $-\infty$ in order to avoid transient processes which decay with the relaxation time constant T_r. T_r is a measure of how quickly charge carriers can move in response to the excitation electric field. The factors that affect T_r are the resistivities of the media internal to (ρ_i) and external to (ρ_e) the membrane, the specific membrane resistivity, R_m and the specific membrane capacitance, C_m. T_r is evaluated

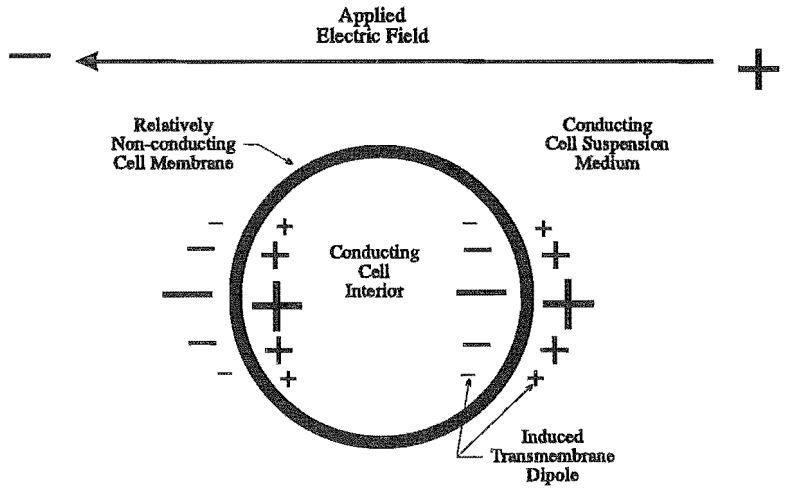


Figure 2.1 Induced dipole orientation for the given applied electric field direction and dielectric conditions.

as [Zimmermann 1986],

$$T_r = \frac{aR_mC_m(\rho_i + 0.5\rho_e)}{a(\rho_i + 0.5\rho_e) + R_m} \tag{2.2}$$

R_m is usually more than two orders of magnitude larger than $a(\rho_i+0.5\rho_e)$ for biological cells. Therefore, Equation 2.2 can be approximated by,

$$T_r = aC_m(\rho_i + 0.5\rho_e) \tag{2.3}$$

A transmembrane potential, $U_m(t)$, is produced by $M(t)$ and is defined as [Holzapfel *et al.* 1982],

$$U_m(t) = \frac{3}{4\pi\epsilon_0 a^3}(M(t)a\cos\phi) \tag{2.4}$$

where ϕ is the angle from a point on the membrane surface to the axis which is parallel to the applied electric field and passes through the origin of the cell (assuming the cell is spherical).

If a constant d.c. electric field, $E(t) = E_p$, is applied to the cell at time $t = 0$, the analytical solution to Equation 2.4 is [Holzapfel *et al.* 1982],

$$U_m(t) = -1.5(1 - e^{\left(\frac{-t}{T_r}\right)})E_p a\cos\phi \tag{2.5}$$

In steady state, $U_m(t)$ is directly proportional to a. Steady state conditions usually hold for applied electric fields lasting longer than about 50μs.

It has been consistently shown that for electric field application times less than

about 50ms in duration, a transmembrane potential of around 1V is required for threshold dielectric breakdown [Chang *et al.* 1992b, Neumann *et al.* 1989]. Exceeding this threshold or critical potential causes substantial amplification of the permeabilization processes. Electric field application times lasting longer than about 50ms produce lower breakdown threshold transmembrane potentials [Teissie and Rols 1993]. It is postulated that variations in electromechanical and polarization mechanisms account for this observation.

2.2.2 The Inherent Transmembrane Potential Effect On Membrane Dielectric Breakdown

Many cells have an inherent transmembrane potential of about 70mV. This potential is less than one tenth of that required for dielectric breakdown of the membrane in most electrofusion and electroporation applications. Membrane breakdown can occur at lower potentials for longer pulse durations [Teissie and Rols 1993]. However, due to the sharp threshold characteristic of membrane breakdown, it can determine whether breakdown occurs or not. If an applied electric field produces a transmembrane potential of the same orientation to the inherent potential, then they add. Conversely, if the applied field produces a transmembrane potential in the opposite orientation to the inherent potential, then they subtract. Both of these conditions apply in a cell. Depending on the direction of the applied electric field, one pole will add whilst the other subtracts. Thus one pole is likely to experience dielectric breakdown at an applied potential 140mV lower than the other pole. This is known as asymmetrical breakdown.

2.2.3 Effective Breakdown Area Of Non-Spherical Cells

Equation 2.4 and Equation 2.5 assume that the cell is a spheroid. In many instances, this is not the case. Cells can be rod, elliptical, spiral, torroidal and even asymmetrically shaped [Curtis and Barnes 1989b]. The $a\cos\phi$ term then no longer holds and a different coordinate system has to be calculated for each cell shape. Since no shape, apart from a sphere, is completely symmetrical through any plane in three dimensions, there exists a statistical variance in the effective area that will experience electropermeabilization if the cells are randomly oriented. Also, at some particular orientations relative to the electric field direction, a non-spherical cell will experience maximal or minimal permeabilization.

Envisaging a rod shaped cell as a cylinder capped with hemispheres, the orientation which exposes the maximum surface area to the electric field is when the long axis of the cell is perpendicular to the electric field. Conversely, if the long axis is parallel to the electric field, the cell will offer the minimum surface area that may be electropermeabilized. A simple model of a rod shaped cell is described in Appendix A. This model estimates that for a cell type that has a length to radius ratio of about 4:1, the

ratio of area that could experience electropermeabilization if all the cells were oriented perpendicular to the electric field to a random orientation of cells is about 5 or 6:1.

2.2.4 Electropermeabilization Dynamics

It is generally perceived that the time course of action in electropermeabilization can be viewed as follows [Neumann 1989]:

$$E(t) \Rightarrow \Delta U_m(t) \Rightarrow \Delta \Xi(t) \tag{2.6}$$

Thus an applied electric field, $E(t)$, induces a change in transmembrane potential, $\Delta U_m(t)$, which in turn induces a change in membrane structure, $\Delta \Xi(t)$. While applying an electric field that produces a transmembrane potential of about 1V will result in a change in membrane structure perceived as a substantial increase in permeability, maintaining the applied electric field amplifies the permeability. A second threshold may then be reached.

Structural rearrangements generated by the applied electric field have an energy relaxation flip characteristic. If the applied electric field is removed or sufficiently reduced before a certain time, it is energetically more favourable for the structurally perturbed membrane to return to its original state. This is known as reversible membrane breakdown [Zimmerman 1982]. However, if the applied electric field remains past that certain time it becomes energetically more favourable, even if the applied electric field is subsequently removed, for the membrane perturbations to continue increasing. Eventually this leads to membrane rupture and loss of cell viability. This is known as irreversible breakdown [Zimmermann 1986].

It has been shown that electropermeabilization using lower magnitude, longer duration electric fields result in lower electroporation efficiencies [Chang *et al.* 1992b]. This indicates that significantly different mechanisms are involved. It has been suggested [Teissie and Rols 1993] these are probably thermal effects not experienced with shorter electropermeabilization pulses.

Many models have been developed to account for electropermeabilization dynamics [Dimitrov 1984, Zimmermann 1986, Neumann *et al.* 1989, Chang *et al.* 1992b]. Most of these incorporate the concept of hole or pore formation within the membrane. Recent physical studies have shown that this concept may be well founded although rather simplistic [Chang 1992]. Some models also tend to predict physical data quite well [Pawlowski *et al.* 1993, Neumann *et al.* 1989]. Even so, the actual mechanisms which contribute to the inception of pore formation are still relatively unknown.

2.3 PHYSICAL DIELECTRIC SPHEROID MODEL

In order to investigate some of the characteristics of membrane dielectric breakdown, a physical model that is electrically very similar to a biological cell was developed [Gaynor and Bodger 1994c].

Essentially the model consists of a thin latex rubber membrane spheroid filled with water of a given conductivity. Due to the similarity of the model to the common balloon, it will forthwith be referred to as the 'balloon' model for convenience and to aid in system conceptualisation. The balloon model is then suspended in a water filled tank, again with a given conductivity, between parallel plate electrodes. High voltage capacitive discharge impulses are then applied to the electrodes which generate electric fields with similar magnitudes to those used in electroporation.

Due to the dielectric nature of the balloon model membrane and the conductive states of the interior liquid and external suspension medium, Equation 2.1 to Equation 2.5 also apply to this system. The radius to membrane thickness ratio is similar to that of biological cells. Thus, the dipole induced in the model membrane will generate transmembrane electric fields with similar magnitudes to those generated in biological cells under electroporation conditions.

2.3.1 Balloon Model Experimental Setup

A latex rubber balloon was filled with domestic water of resistivity 100Ωm until the membrane was under tension relative to its unstretched state. Its expanded diameter was approximately 120mm. The balloon was then suspended in a polyethylene tank filled from the same water source. This is shown in Figure 2.2. The perspex suspension rod was required to maintain the balloon in a static position and provide electrical insulation.

The tank diameter was 800mm. Two 340mm by 480mm parallel plate electrodes spaced 500mm apart were suspended into the tank such that the balloon model occupied a space between the electrodes. The tank diameter was sufficiently large so that any electric field established between the electrodes was relatively uniform and not significantly influenced by the tank sides. The electrode spacing also allowed for the desired electric field magnitudes with the apparatus available.

One of the electrodes was connected to a ground plane while the other was connected to the third stage output of a 14 stage, variable 1.4MV, inverted Marx impulse generator as used in a high voltage testing laboratory for impulse testing of electric power transformers [Khalifa 1990]. An impulse was then applied to the electrode, tank and balloon system with the generator.

The result of the applied impulse in the water tank balloon apparatus is that dielectric breakdowns of the latex rubber material exposed to this field occur at points

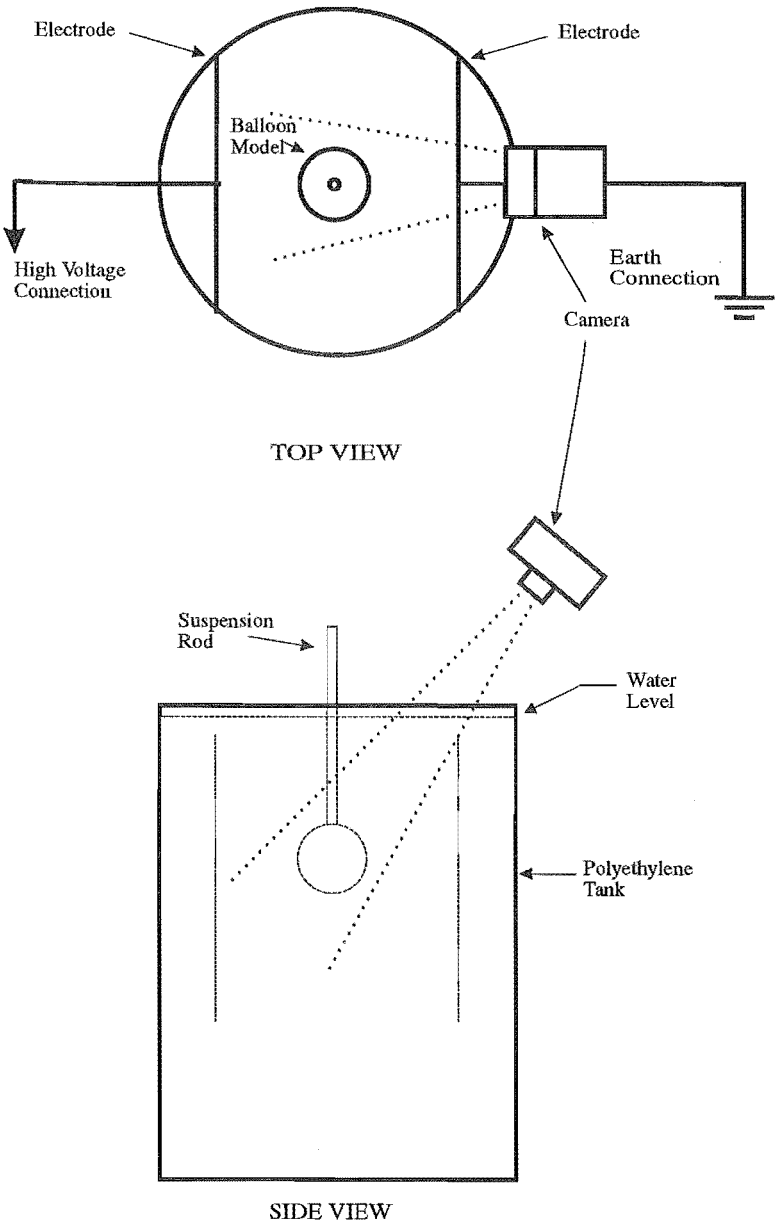


Figure 2.2 Balloon model experimental setup.

of weakest dielectric strength.

The peak voltage and electric field magnitudes and $1/e$ decay time constants were all measured to an accuracy of $\pm 5\%$.

2.3.2 Balloon Model Experimental Results

The applied impulses had a peak magnitude ranging between 90 and 200kV and its shape was determined as having a 1μs rise time and an 8μs $1/e$ decay time. Figure 2.3 shows an oscillograph of an actual capacitive discharge waveform with a peak voltage of 98kV. The magnitude of the effective uniform electric field between the 500mm electrodes was between 1.8kV/cm to 4.0kV/cm which is of the same magnitude as that used in the electroporation of many eukaryotic cell types [Neumann *et al.* 1989, Chang *et al.* 1992b]. For the experimental impulse tests the electric field across the membrane equated to be between 4.8MV/cm and 24MV/cm. These are of the order of the dielectric strength of solid dielectrics. Typically the very best intrinsic breakdown strength of solid dielectrics as determined in physics laboratories [Alston 1968] is 20MV/cm. However, the practical design field strengths of solid dielectrics used in the electric power industry are very much lower. They are of the order of 2MV/cm which recognizes the reduction of dielectric strength due to surface imperfections. With a latex rubber balloon, the surface is unmachined and design strengths are most likely to apply.

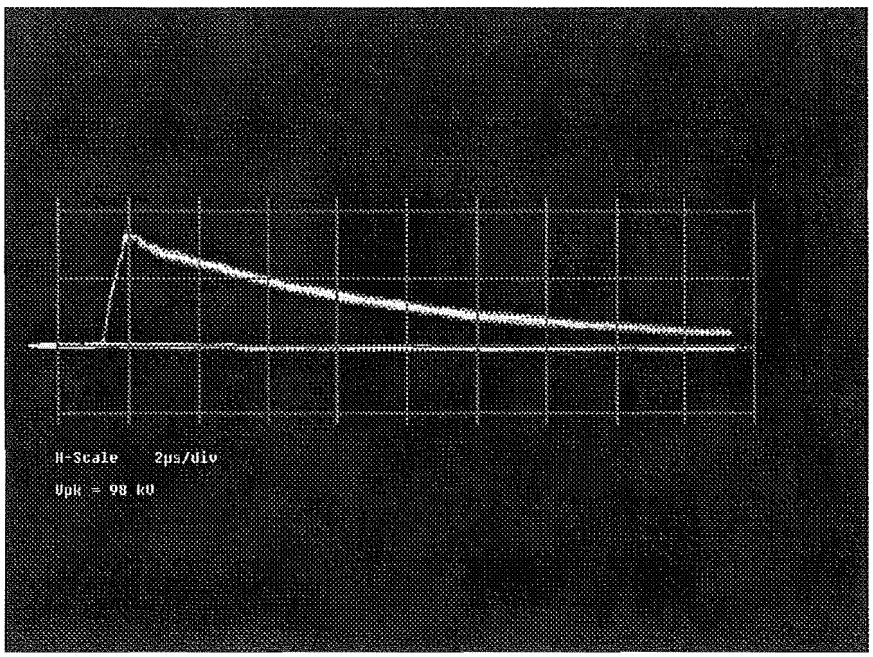


Figure 2.3 Oscillograph of a typical impulse voltage waveform used in the experimentation.

2.3.3 Water Droplet Formations

When the impulsed balloon was removed from the tank it was apparent that a number of holes had been formed in the rubber membrane as shown in Figure 2.4. Droplets of water formed on the outside of the balloon membrane after it had been towel dried. Repeated drying produced the same droplet formation. It was concluded that the membrane was perforated in a large number of places, but each perforation was insufficient in size to cause the balloon to undergo spontaneous mechanical fracture.

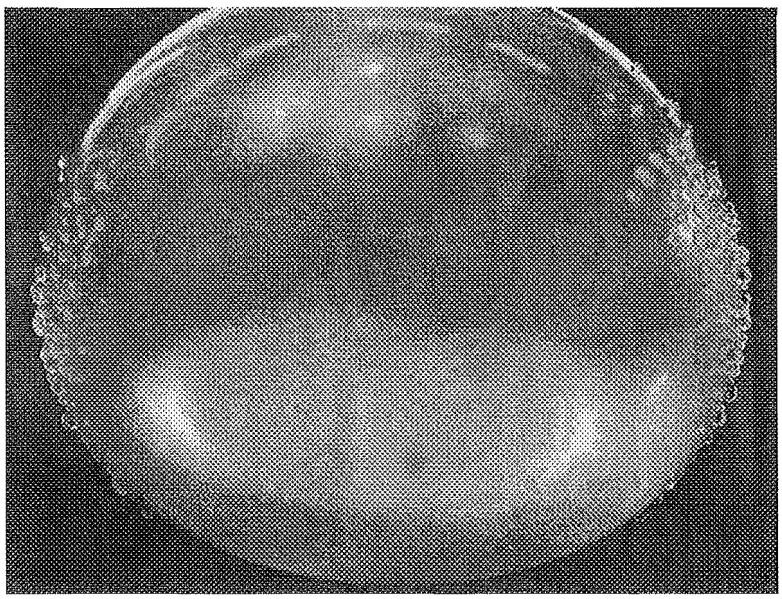


Figure 2.4 Water droplet formation on the pre-dried surface of an impulsed balloon model.

The perforations produced did so on the poles of the balloon that were facing the electrodes in the tank setup. There was also evidence, by the relative amount of droplet formation, that there were more holes formed on one side relative to the other. In other words, asymmetrical breakdown was observed. This is due to the surface charge that is transferred to the balloon model during setup. The surface charge produces an inherent transmembrane potential much the same as a biological cell.

A voltage threshold effect was observed for the production of holes. Below about 135kV very little or no droplet formation was observed, even after multiple impulses. Above this threshold, multiple holes were apparent after a single impulse. Also, above the threshold voltage, multiple impulsing on a single balloon produced successively increasing hole numbers on the pole faces as indicated by increasing droplet density.

These features of hole formation mimic those that are expected and cited for electroporation in the biological sense [Neumann *et al.* 1989, Chang *et al.* 1992b].

2.3.4 Photographic Photon Capture

If the breakdown of the balloon membrane was an ionisation dielectric breakdown then such a breakdown would be accompanied by an emission of light from the arc so formed. Photographic emulsions are available which can detect very small emissions. The balloon apparatus was photographed in a completely darkened environment, so determined by a nil exposure of Konica 3200ASA colour film after 30 seconds. The camera position is shown in Figure 2.2. It was placed over the earthed electrode for electromagnetic shielding and safety purposes. Due to this position only the low voltage pole face can be seen or photographed.

A series of impulses was applied to the balloon whilst the camera shutter was open. The resulting photographs show a faint but distinct capture of light confirming an ionisation or arc source. These images were digitally scanned and enhanced to increase their visual contrast relative to the background. The enhancement is required to eliminate previously experienced problems of losing definition in reproducing the images on paper (see [Gaynor and Bodger 1994c]). Figure 2.5 shows the light emitted from a series of tests. The image blurring is caused by the impulse generated balloon expansion and elongation parallel to the field direction which is also observed in biological cells during impulsing [Itoh *et al.* 1990]. The expansion is transient and only lasts about as long as the impulse.

Figure 2.5(a) is an image showing the ionisation in the membrane due to a single, 3.6kV/cm peak electric field, impulse. A low level background illumination was used to indicate the balloon position. Figure 2.5(b) to 2.5(f) show a series of sequential images of a single balloon undergoing subsequent, 3.0kV/cm peak electric field, impulses. There is an increase in the number of breakdown points for each successive impulse. Also, once a point in the membrane has broken down, it continues to breakdown under further impulsing. The peak electric field threshold was observed to be at about 2.7kV/cm. However, Figure 2.6 shows a sequence of three impulses on a single balloon which starts at 3.6kV/cm and ends with a 2.4kV/cm impulse. The 2.4 kV/cm impulse is well below the previously observed threshold, but now shows a high degree of breakdown. This indicates that once perforated, the breakdown threshold of a particular point is lowered.

2.3.5 Microscope Evaluation

Samples of membrane from the areas known to show water droplets on the impulsed balloon were looked at under an optical microscope. Holes were apparent and looked slightly discoloured a darkened brown from the more translucent parent material suggesting a possible burning in of the holes. Membrane samples were then looked at under a scanning electron microscope which gave significantly more information about hole structure. The electron micrograph of a non-impulsed sample in Figure 2.7(a) shows

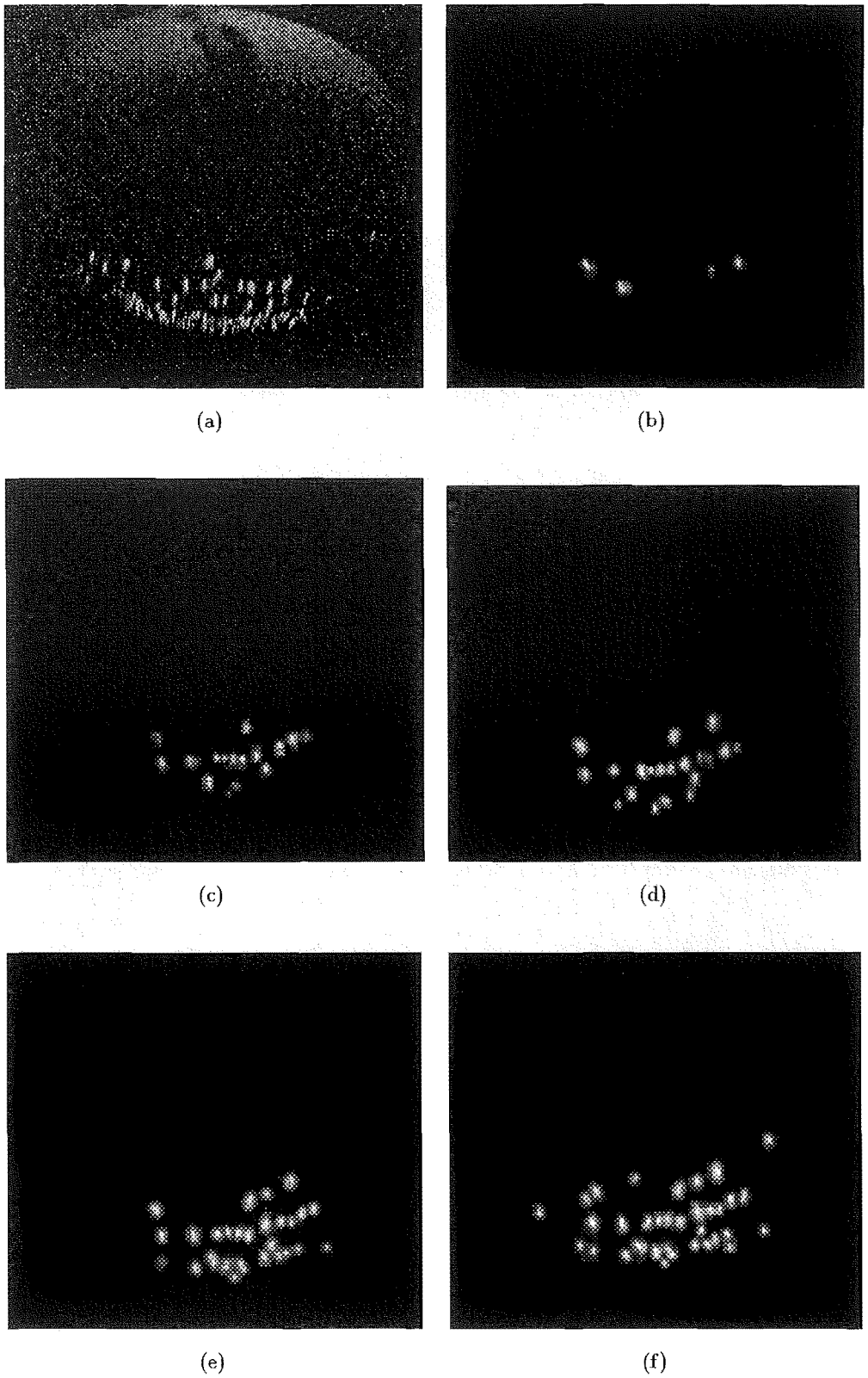


Figure 2.5 Photon emission from the balloon model undergoing impulsing. (a) Photon emission from a single 3.6kV/cm pulse (balloon model spatial placement shown). (b)-(f) Photon emission from consecutive 300kV/cm pulses on a single balloon model.

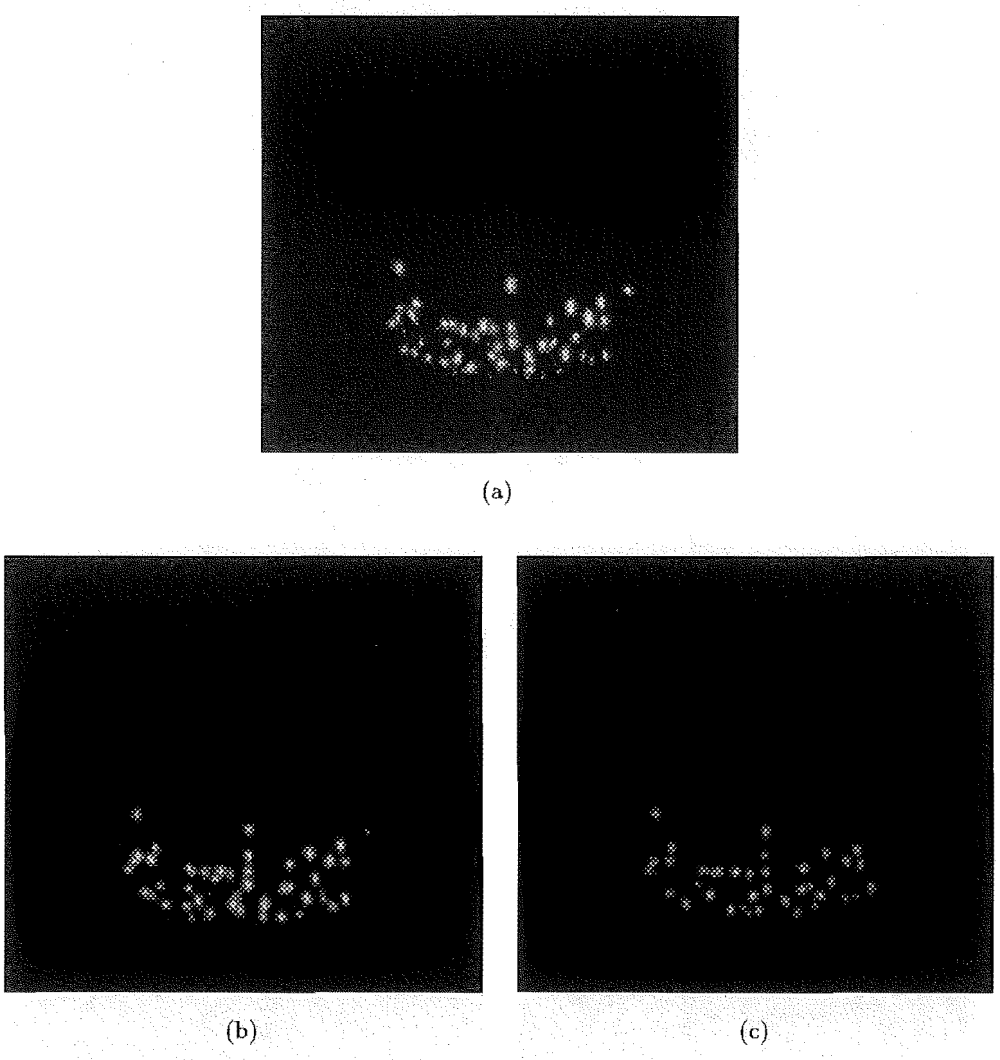


Figure 2.6 Photon emission from consecutive impulses on a single balloon model. (a) 3.6kV/cm. (b) 3.0kV/cm. (c) 2.4kV/cm.

the surface appears to be creatored and porous. The electron micrograph in Figure 2.7(b) shows a typical impulse generated hole at a peak electric field of 3.3kV/cm. The surrounding membrane appears to be more heavily creatored and porous relative to the control.

2.4 DISCUSSION

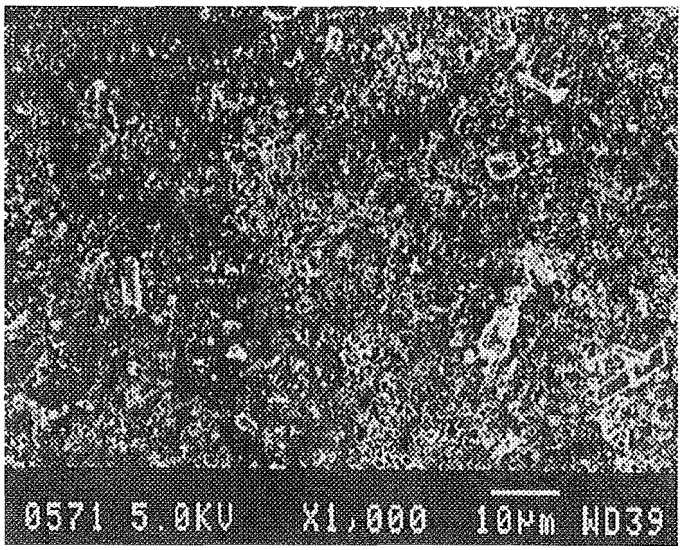
Although not directly observable, it is generally assumed that the initiation of dielectric breakdown in biological membranes is primarily a mechanical molecular rearrangement brought on by electrostatic forces [Kinosita *et al.* 1992]. The electrical similarities of the balloon model to a biological cell indicate that the balloon membrane should experience similar mechanical forces [Gaynor and Bodger 1994c]. Thus, one might expect that the model would exhibit evidence of electromechanical dielectric breakdown. Experimental data does not corroborate this reasoning.

Some general conditions exist which suggest dominant breakdown mechanisms. Of these conditions, photonic emission is a very strong indicator for ionisation or excitation of molecules to a conductive state [Llewellen Jones 1967, Fothergill 1991, Kojima *et al.* 1992]. The absence of photon emission would suggest the breakdown was primarily electromechanical in nature [Fothergill 1991].

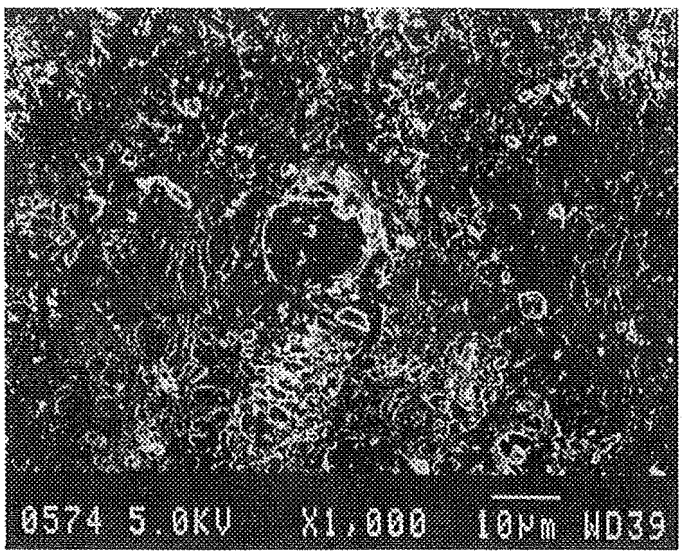
Under similar electrical conditions, different dielectric materials may emit photons which vary in energy depending on the respective material excitation energies. Since the energy of a photon is directly proportional to its wavelength, the emission may produce a light spectrum anywhere between infra red (IR) and ultra violet (UV). The photon emission from the balloon model suggests that the primary mechanism of its membrane dielectric breakdown is molecular ionisation. It is possible that mechanical tearing of the dielectric in the presence of a high electric field could produce the arcing [Donaldson *et al.* 1990]. This is considered unlikely as the reported materials and surrounding media are very different than those used in either the balloon model or biological cell electropermeabilization.

It has been stated [Coster and Zimmermann 1975b] that rubber behaves in a different way to biological membranes. The difference has to do with the relative changes in Young's modulus with temperature. Biological membranes can be modelled as bi-layer phospholipid structures which have hydrophillic surfaces and a hydroscopic centre [Hoppe *et al.* 1983] and contain various forms of proteins and ion channels. Although the proteins and ion channels do affect the dielectric properties of the membrane, the transmembrane potential required for dielectric breakdown is relatively the same for many different cell types [Chang *et al.* 1992b]. This suggests that it is probably the lipid bi-layer that mainly determines the dielectric strength of the membrane.

As determined by the experiments of this chapter, a latex rubber membrane is quite different to a biological cell membrane. It is many molecules in thickness and



(a)



(b)

Figure 2.7 Electron microscope images of a typical balloon model membrane. (a) Non-impulsed. (b) A single 3.0kV/cm impulse generated hole

appears to be arranged of layers of intertwined strands which are porous enough to let air pass through it over time. However, this may allow the balloon membrane to act more like the biological membrane than would be expected. If water were to 'seep' a little way in on both the outer and inner surfaces due to capillary action, this would mimic the hydrophillic - hydroscopic - hydrophillic characteristics of the biological cell. As in a biological membrane, the balloon membrane has numerous local deformities. In dielectric materials, deformities may cause localised electric field enhancement by up to three or four orders of magnitude [Alston 1968, Fothergill 1991, Khalifa 1990] which aids in the initiation of dielectric breakdown. Although the balloon membrane is a solid, it is also an elastomer which in many ways mechanically models the biological membrane. Whatever the detailed structure of the actual membranes of biological cells and balloons, the results suggest that their behaviour under electric impulse stimulation is similar.

It remains to repeat the experiments which show definite ionisation characteristics, on actual biological cells. As previously stated, the wavelength of any possible photon emission due to ionisation could lie anywhere between IR to UV. Hence this entire spectrum must be investigated before a more definitive conclusion can be made on the mechanisms of biological membrane breakdown. Preliminary experimentation indicates that there is very little or no photon emission from biological cell membranes in the visible region of the spectrum.

In Section 2.3.4, it was shown that once a point in the membrane has exhibited dielectric breakdown, it will continue to breakdown at previously sub-breakdown threshold electric field magnitudes. This is an interesting aspect of the general electroporation system which does not appear to have been considered for multiple impulse electroporation applications.

At present, to obtain hole duration of minutes so that long chain molecules can transverse cell membranes, the temperature of the cells must be lowered and/or multiple impulses must be applied over time [Zimmermann 1986, Neumann *et al.* 1989, Chang *et al.* 1992b]. As the temperature lowers, the corresponding peak electric field impulse magnitude required for optimum electropermeabilization rises. Depending on the apparatus being used, and/or the electrode geometry, generating higher magnitude electric fields may either be beyond the apparatus design limits or cause flashover conditions at the electrodes. Also, when a cell is impulsed a number of times in a time scale of minutes at an electric field intensity that causes permeabilization, the probability of the cell remaining viable is correspondingly reduced. As indicated in Section 2.3.4, this is due to the progressive increase of hole density and size with each successive impulse, which eventually causes irreversible membrane damage. After an initial optimum permeabilization impulse, successive impulse electric field magnitudes could be reduced so that breakdown only occurs in existing pores. Thus, it may be possible to maintain permeability via impulses without compromising cell viability. This eliminates the need

to reduce cell temperature and increase impulse electric field magnitude.

In some electroporation applications it is critical that the possible transport of material is maximised [Inoue *et al.* 1990]. In these cases, it is important to electropermeabilize the largest possible cell surface area, yet maintain cell viability. Often, non-spherical cells and especially rod shaped cells are used for experimentation. In Section 2.2.3, it was shown that for rod shaped cells of a particular morphology, the surface area that can be electropermeabilized is increased 5 or 6 times if the cells are all oriented perpendicular to the applied electric field. Thus, in some instances, it may be desirable to align the cells prior to electropermeabilization. Research has shown [Iglesias *et al.* 1989, Zimmerman 1982] that it is possible to align rod shaped cells either parallel or perpendicular to an applied a.c. electric field of moderate magnitude (around 10^2V/cm). The direction of alignment is dependent on factors such as the dielectric properties of the suspension medium, cell membrane and cell contents and electric field frequency. Other cell shapes are likely to exhibit at least some kind of alignment characteristic which may or may not expose the maximum surface area that may be electropermeabilized.

If the surface area that undergoes electropermeabilization is trying to be maximised, then asymmetrical breakdown is a counterproductive characteristic. It has been shown that under multiple pulse electroporation, reversing the polarity between successive pulses can eliminate this problem [Tekle *et al.* 1991]. Cell viability and transfectant yield is also increased. The other benefits of using a bi-polar electric field to induce electropermeabilization will be discussed in greater detail in Chapter 3.

2.5 CONCLUSIONS

Previous theoretical and physical models of electroporation have greatly contributed to understanding the mechanisms involved with and predicting behaviour in, real systems. However, due to the nanoscopic scale involved, it has been difficult to visualise aspects such as pore distribution, density, morphology and dynamics.

A rubber balloon model has been proposed as being representative in its behaviour of the breakdown of biological cells under electric impulse conditions. Support to this proposal has come from the consideration of the intensity of the membrane electric field strength and the general morphology of the models such as diameter to membrane thickness ratios, local membrane deformities and elasticity.

Electric arcs exhibit heat and light. The effects of each have been observed using an electron microscope to visually compare hole edges to controls and through the photographic capture of emitted photons.

The experimentation on the rubber balloon membranes does not quantify the detailed mechanism of breakdown. However, they do indicate that there are global similarities between the balloon model and biological cells under impulse conditions. The

results lend weight to the observation that the initial breakdown mechanism may indeed be an ionisation in nature and not electromechanic. They also suggest that conventional electroporation procedure modifications be made when using multiple impulses, especially with the reduction in applied electric field magnitude.

When high efficiency electroporation is required, it is important to consider as many aspects, biological and physical, as possible. Some significant features such as cell shape and asymmetrical breakdown are often overlooked. The potential benefits of alignment and alternating pulse polarity warrant the extra effort required to test these options.

Chapter 3

ELECTROFUSION

3.1 INTRODUCTION

Ever since Cesar Milstein and Georges Kohler developed the first reliable technique of fusing cells for antibody research in 1975 [Kohler and Milstein 1975], considerable effort has been made to optimise the act of producing hybrid cells (hybridomas) [Curtis and Barnes 1989c]. The principle reason for this effort is due to the very important research concerned with monoclonal antibodies [Chien and Silverstein 1993]. Worldwide confirmation on the research importance has been shown in awarding Milstein and Kohler the Nobel prize in 1984. Areas of monoclonal antibody research are widespread and include a great deal of immunology [Roitt *et al.* 1985, Steenbakkers *et al.* 1993, Zimmermann *et al.* 1990], cancer [Vollmers *et al.* 1993, Klock *et al.* 1992] and AIDS [Greene 1993] investigation. There are other important applications of electrofusion, such as plant cell fusion to facilitate gene transfer [Zimmermann and Scheurich 1981].

Soon after 1975, the most popular process for fusing cells involved chemically treating the cell membranes with polyethylene glycol (PEG), making them highly permeable [Taggart and Samloff 1977]. This is a fundamental state required for cell to cell fusion. However, around 1978 it was found that cell fusion could be accomplished by a largely electrical technique. At this time the term 'electrofusion' was coined. Considerable interest was shown in electrofusion and the similar electrical process, electroporation, which has led to a similarly large amount of theory [Zimmermann 1986, Hofmann and Evans 1986, Weaver and Powell 1987, Neumann *et al.* 1989, Chang *et al.* 1992b]. Due to the physical nature of the biological aspect of these processes, most of the theory deals with electrical models for the cells and their membranes.

Although current electrofusion techniques can produce hybridoma yields more than an order of magnitude greater than chemical means [Foung *et al.* 1990], there is still room for improvement. Making the electrofusion process more efficient can only increase its versatility as a tool in biotechnological research.

To enable fusion between cells, their membranes must be in close contact and be in a state of high permeability. Both of these criterion can be induced through electrical

stimulation. Close cell to cell contact is most commonly achieved by utilising an effect known as dielectrophoresis [Pohl 1978, Pethig *et al.* 1992, MacDonald *et al.* 1992]. Other methods of producing close cell-cell contact are also used. They may include centrifuge [Abidor *et al.* 1993] acoustic [Bardsley *et al.* 1989] or chemical [Teissie and Rols 1986] means. The high permeability state is caused by high magnitude electric fields (kV/cm) of short duration (μs-ms) [Neumann *et al.* 1989, Chang *et al.* 1992b]. These electric fields are effectively the same as used in electroporation. The high electric field produces transmembrane potentials that lead to dielectric breakdown of the membrane. This creates many micropores and a state of high permeability. Two membranes in close contact which are highly permeable will tend to fuse.

There are factors that complicate electrical fusion of cells which must be considered in order to optimise fusion yields. Close cell contact brought about by dielectrophoresis is relatively well understood and simple to implement [Pohl 1978, Chang *et al.* 1992b, MacDonald *et al.* 1992]. Yet careful considerations have to be made for each application. Electropermeabilization of the membrane is more complicated and hence contributes a number of the complicating factors in electrofusion.

In this chapter, dielectrophoresis, electropermeabilization and the biological environment are investigated in an attempt to offer well founded information on how the electrofusion process can be improved. Also, due to the common electropermeabilization factor, relevant aspects are considered for the electroporation process. Owing to its relative importance, most of the chapter is dedicated to electropermeabilization characteristics in electrofusion.

3.2 DIELECTROPHORESIS

The field of dielectrophoresis is as complicated and extensive as electrofusion and electroporation put together [Pohl 1978, Pethig *et al.* 1992, Neumann *et al.* 1989, Chang *et al.* 1992b]. Thus, only those factors pertaining to the induction of close cell-cell contact will be introduced.

In the most simple terms, dielectrophoresis is the movement of relatively non-conducting or charged particles in a non-uniform a.c. electric field [Pohl 1978]. When dielectric particles are placed in a non-uniform a.c. electric field, a net force is exerted on the particles causing them to move. The force direction and magnitude is dictated by the dielectric permittivities and conductivities of the particle material and the media the particle is suspended in and the frequency, orientation and degree of non-linearity of the applied electric field. Figure 3.1 illustrates the basic properties of dielectrophoresis. Polarization of the membrane due to an applied electric field, leads to a transcellular electric field and charge density product imbalance. If the applied electric field is uniform (Figure 3.1(a)), the generated electrostatic forces are also uniform and cancel out. If the applied electric field is non-uniform (Figure 3.1(b)), then a net force results

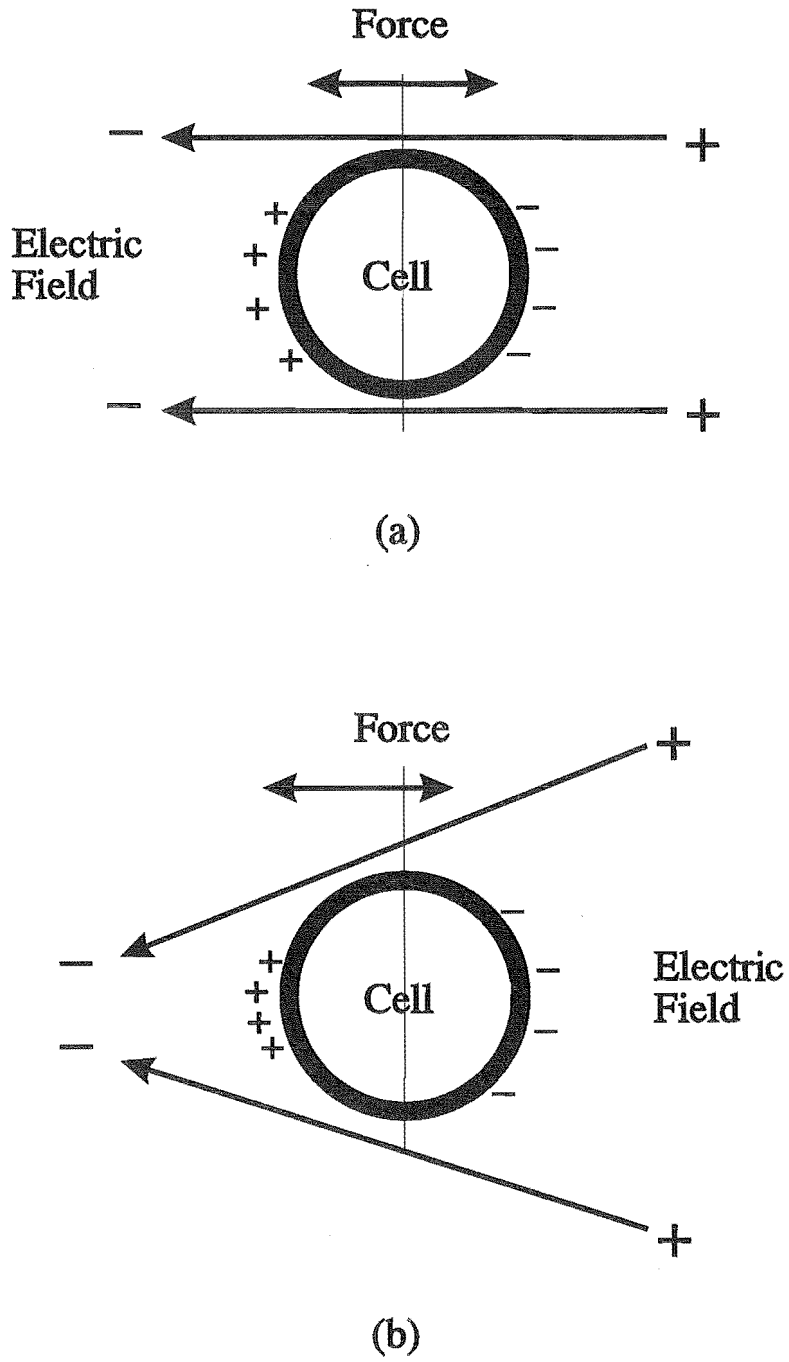


Figure 3.1 Dielectrophoretic forces induced on a cell with the given conditions. (a) Zero net force due to a uniform applied electric field. (b) Net force induced by a non-uniform applied electric field

owing to the enhanced charge density and higher electric field magnitude at one pole. This net force is independent of electric field polarity as the charge distribution reverses according to polarity. The net force direction and magnitude is determined by the dielectric properties of the suspension media and cell membrane and the applied electric field frequency and magnitude. Figure 3.1(b) indicates the most common net force condition for electrofusion applications.

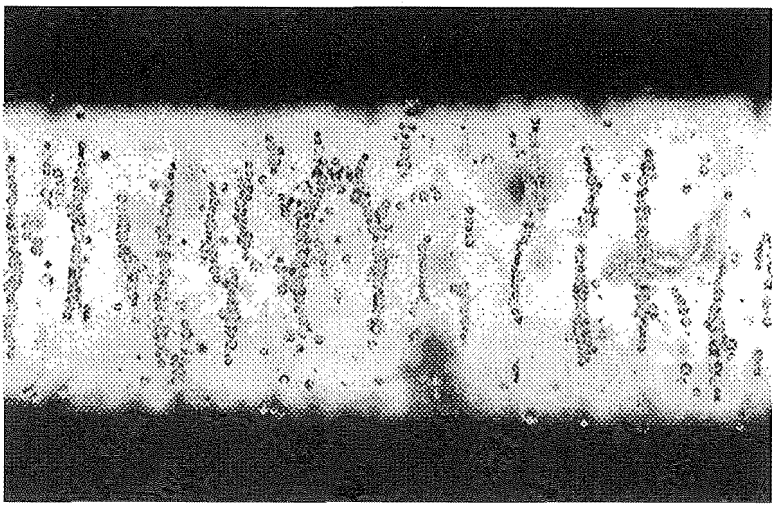


Figure 3.2 Example of pearl chain formation of murine FOX-NY myeloma cells.

If a number of particles are present, then depending on the particle size and density and the electric field magnitude, pearl chain formation of the particles along the electric field lines can be induced [Zimmerman 1982]. An example of pearl chain formation of cells between parallel wire electrodes is shown in Figure 3.2. Close cell-cell contact is experienced in the pearl chains. The spatial orientation of the pearl chains allows the permeabilization pulse to cause permeabilization of the cells in the contact zone. This then triggers membrane fusion. Pearl chain formation occurs due to the dielectric particle inducing local electric field non-linearities that attract relatively close neighbouring particles. As a result of this effect, it is possible to produce pearl chain formation by applying electric fields between parallel plate electrodes if the particle concentration is high enough [Sowers 1992]. This is desirable in electrofusion applications as the electric field magnitude remains quite constant between the electrodes which is important when applying a permeabilization pulse at an optimum electric field magnitude. However, most electrofusions are carried out between parallel wire electrodes. These produce very effective pearl chain formation but also result in a variation of electric field magnitude. Optimum permeabilization electric field magnitudes can then only be experienced in certain regions. Unless all close contacting cells are in those regions, the electrofusion efficiency must be sub-optimal.

In most applications, particularly monoclonal antibody research, it has been found that a symmetrical a.c. electric field with a frequency around 1MHz and a magnitude in the 10^2V/cm range, induces satisfactory pearl chain formation without generating too much heat in the suspension (fusion) media [Chang *et al.* 1992b].

Application times of the dielectrophoresis electric field prior to the electropermeabilization pulse are determined primarily on how long it takes for virtually all cells to be forming pearl chains at least two cells in length. The alignment field is again applied after the permeabilization pulse or pulses for about 30s to keep the permeabilized membranes in close contact. Fusion of the membranes has usually initiated by that time.

3.3 THE INDUCED TRANSMEMBRANE POTENTIAL

In Chapter 2, Equation 2.1 to Equation 2.5 were shown to describe the induced transmembrane potential of a cell from an applied electric field. In electrofusion procedures the same equations hold true. Although slightly greater transmembrane potentials seem to be needed to initiate optimum electrofusion compared to optimum electroporation. This may indicate that different mechanisms are involved [Teissie and Rols 1993].

In electroporation, most of the cell population is usually of a homogeneous size. Therefore, an applied electric field would generally produce the same transmembrane potential over most of the cell population. Hence, when an optimum electropermeabilization potential is applied, most of the cells react in a similar fashion. However, in many electrofusion applications, cells of significantly different radii are often required to be fused [Zimmermann 1986, Chang *et al.* 1992a].

In order to induce fusion between cells, both membranes that are in close contact must experience dielectric breakdown which makes them highly permeable [Sowers 1992]. Due to the radius dependence of transmembrane potential to a conventional d.c. pulse lasting longer than about 10μs shown in Equation 2.5, excitation electric field magnitudes that cause breakdown in the small radius cells are used. These electric fields can cause irreversible membrane breakdown in the large cells. If a significant number of the large cells are lysed, fusion yields are severely reduced.

The solution of Equation 2.4 to an applied sinusoidal excitation electric field, $E(t) = E_0 \cos \omega t$, is [Holzapfel *et al.* 1982],

$$U_m(t) = \frac{1.5 E_0 a \cos \phi}{1 + (\omega T_r)^2} (\cos \omega t + \omega T_r \sin \omega t) \tag{3.1}$$

where E_0 is the peak magnitude of the applied electric field and ω is the angular frequency of the applied electric field. Equation 3.1 has the amplitude, U_{m0},

$$U_{m0} = \frac{1.5 E_0 a \cos \phi}{\sqrt{1 + (\omega T_r)^2}} \tag{3.2}$$

If $(\omega T_r)^2$ is about an order of magnitude larger than 1, Equation 3.2 can be approximated by,

$$U_{m0} = \frac{1.5 E_0 a \cos\phi}{\omega T_r} \tag{3.3}$$

Using the expression for T_r given in Equation 2.3, Equation 3.3 can in turn be simplified to,

$$U_{m0} = \frac{1.5 E_0 \cos\phi}{\omega C_m(\rho_i + 0.5\rho_e)} \tag{3.4}$$

Equation 3.4 is independent of radius. Therefore, if either the frequency or relaxation time constant are large enough, the radius dependence of transmembrane potential and hence dielectric breakdown is eliminated.

Fourier analysis of d.c. pulses shows that they can be thought of as a sum of sinusoidal waveforms [Zimmermann 1986]. As the pulse duration shortens, the frequency of the sinusoidal waves increase. This suggests that for a short enough pulse, the frequency components may be high enough to satisfy the requirements of Equation 3.4.

As previously stated, the electric pulses used in conventional electrofusion are in the form of rectangular or capacitive discharge d.c. impulses. The d.c. nature of these impulses contributes to two additional problems. The first is the result of asymmetrical breakdown due to the inherent transmembrane potential. If the side of a cell in close contact with another cell does not breakdown due to the impulse, then fusion cannot be initiated even if the other cell does breakdown [Zimmerman 1982, Zimmermann 1986]. The second problem is caused by the rotation of cells when exposed to d.c. impulses followed by a.c. electric fields [Zimmerman 1982, Holzapfel *et al.* 1982]. The rotation effectively breaks close contact and moves the high permeability area away from the contact site. After a few milliseconds, most cells tend to return to their original orientation. However, the probability of fusion is reduced. Solving these particular problems may increase fusion yields in many instances.

3.3.1 Numerical Model Of Induced Transmembrane Potential

Since the transmembrane potential plays such a major role in electropermeabilization, a numerical computer model was devised to simulate this potential over time [Gaynor and Bodger 1994b]. The aim of the model was to look at how various electric field configurations affect the transmembrane potential, lending insight to solving problems in electrofusion. Some assumptions have been made, including that the cells are spherical and that the dipole reaction in the membrane is passive.

The model works out the transmembrane potential primarily from Equation 2.4. The integration technique is numerical and thus allows the expression of excitation elec-

tric fields which may make the integral very difficult or impossible to solve analytically. Since the lower limit cannot be taken at $-\infty$, it is taken at time $t = 0$. Transmembrane potential figures are given at the polar points, i.e., where $\cos\phi = 1$.

3.3.2 Model Simulation Results

To validate the accuracy of the simulation results, the model with its numerical integration was initially tested against known analytical solutions. All constants used in the simulations were indicative of a real electrofusion system and were obtained from [Zimmermann 1986].

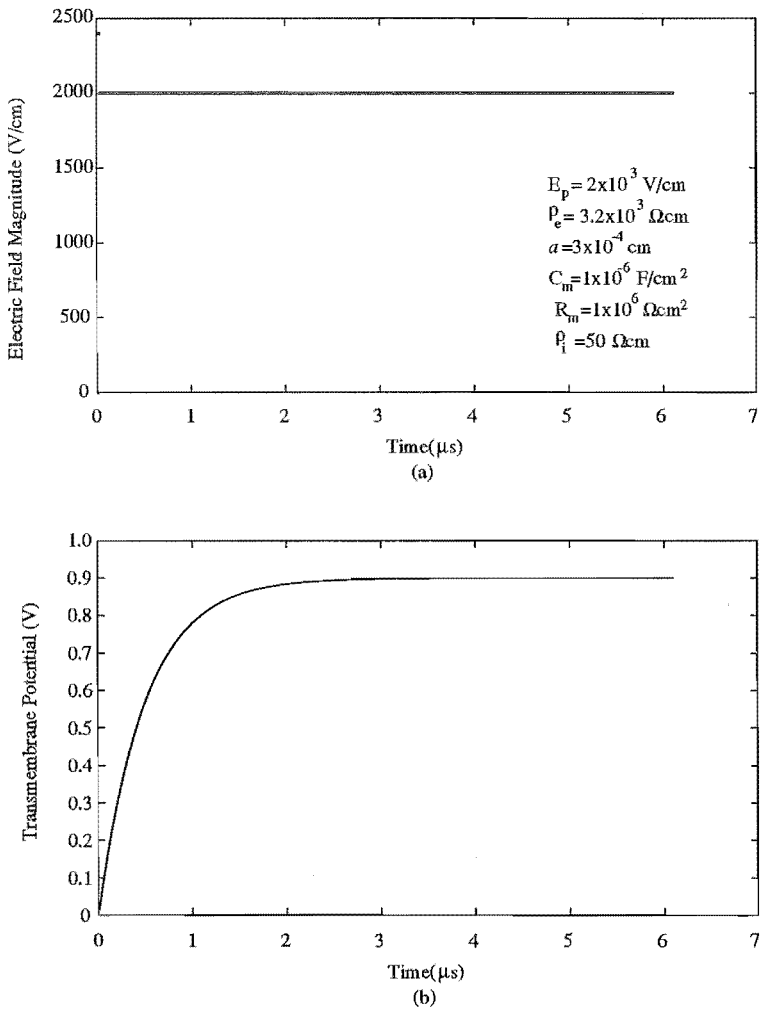


Figure 3.3 (a) Constant applied electric field. (b) Superposition of the analytical and numerical solutions for the induced transmembrane potential.

For a constant electric field applied at time $t = 0$, Equation 2.5 holds. Figure 3.3(a) shows the excitation field. Figure 3.3(b) shows the analytical solution superimposed on

the numerical model solution for the induced transmembrane potential. Only one line is apparent as the model follows the analytical solution so closely. All the constants used for the equations are shown in the figure.

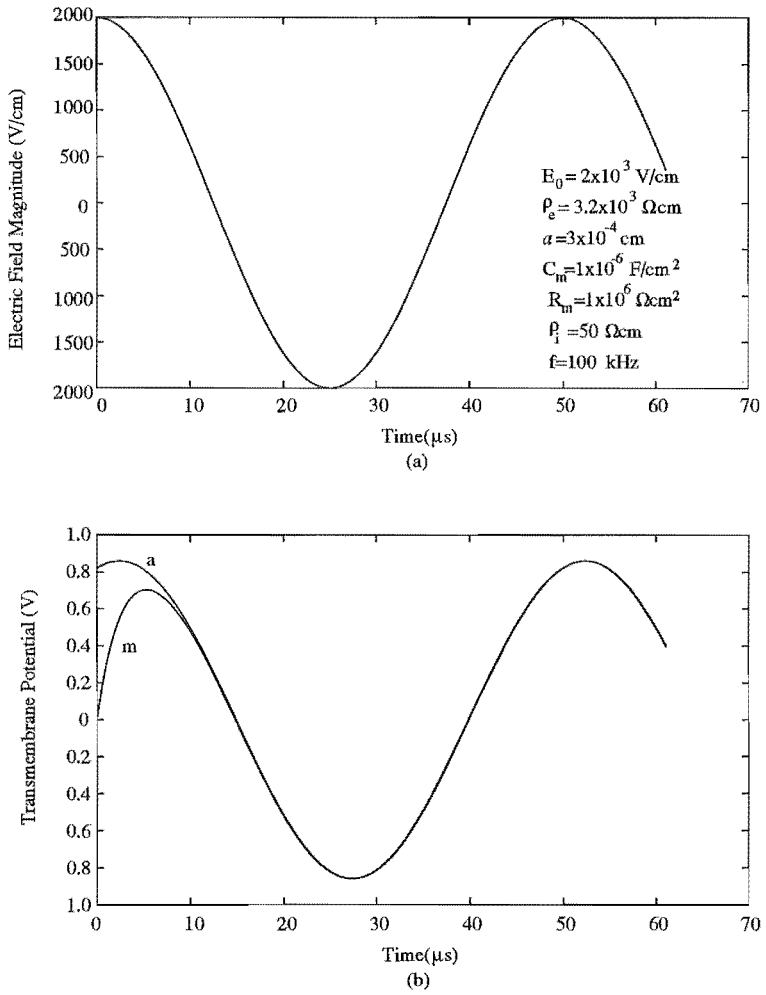


Figure 3.4 (a) 100kHz sinusoidal applied electric field. (b) Superposition of the analytical (a) and numerical model (m) solutions for the induced transmembrane potential.

For a sinusoidal excitation electric field, applied at time $t = -\infty$, Equation 3.1 applies. Figure 3.4(a) shows a sinusoidal excitation field with a frequency of 100kHz. Figure 3.4(b) shows the analytical solution superimposed on the model solution. Since the analytical solution takes the applied field at $t = -\infty$, all transient processes have ended at $t = 0$. The model assumes the field is applied at $t = 0$ so the transient rise in potential is apparent. This is effectively what happens in a real situation when a sinusoidal field is applied to the cell. The steady state model solution follows the analytical solution very closely.

The solution to capacitive discharge or rectangular electric field impulses is diffi-

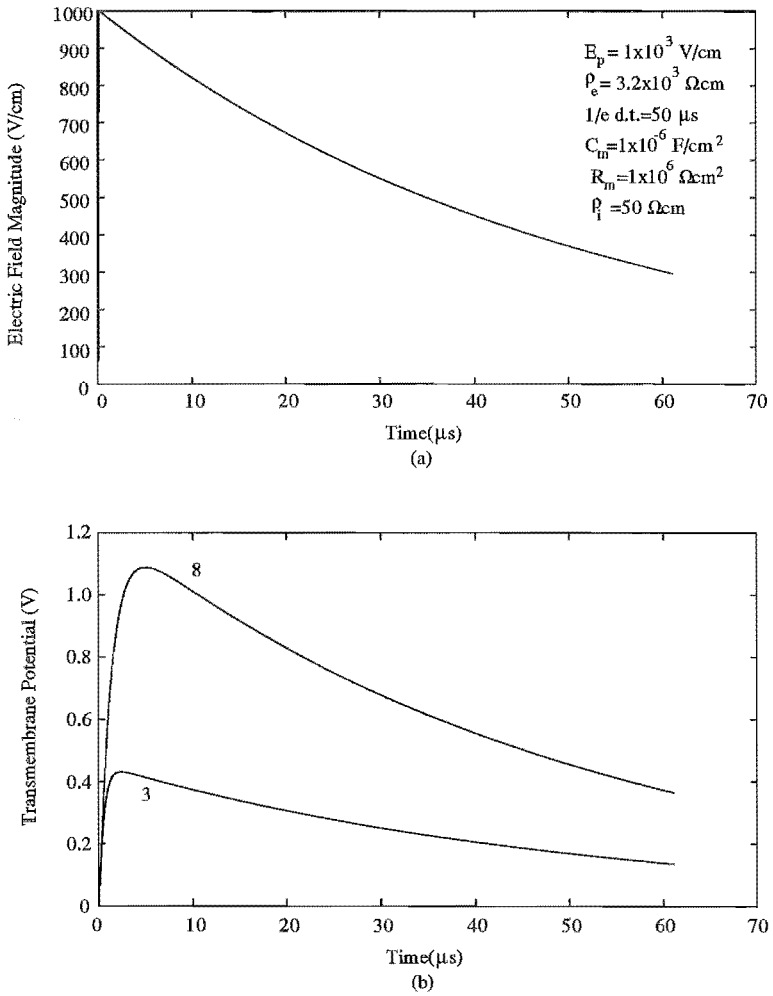


Figure 3.5 (a) 50μs $1/e$ decay time constant capacitive discharge impulse applied electric field. (b) Induced transmembrane potentials for 8μm (8) and 3μm (3) radius cells.

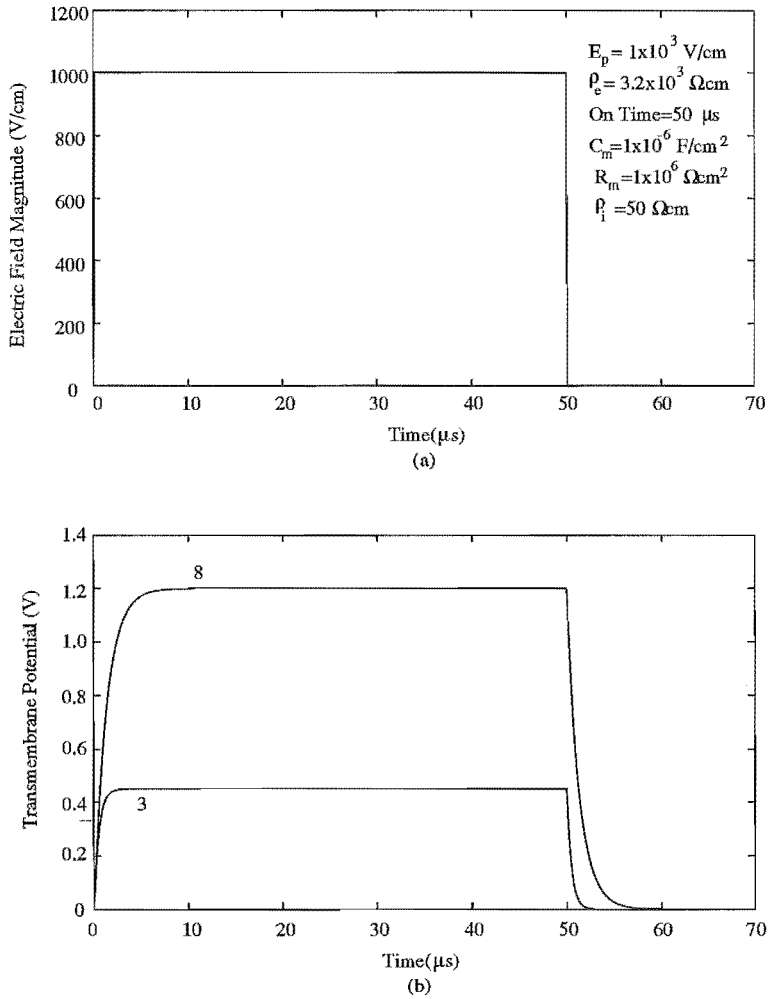


Figure 3.6 (a) 50μs on time rectangular impulse applied electric field. (b) Induced transmembrane potentials.

cult to obtain analytically. However, using the numerical model, Figure 3.5 shows a capacitive discharge excitation field and the corresponding transmembrane potentials for 3μm and 8μm radius cells. The discharge has a $1/e$ decay time of 50μs and a peak magnitude, E_p, of 1×10^3V/cm. Figure 3.6 similarly shows a rectangular excitation electric field with a 50μs on time and E_p of 1×10^3V/cm and the corresponding transmembrane potentials. The peak potential of the 3μm cells only reach about 37.5% of the 8μm cells. This is the same percentage given by the ratio of small cell radius to large cell radius.

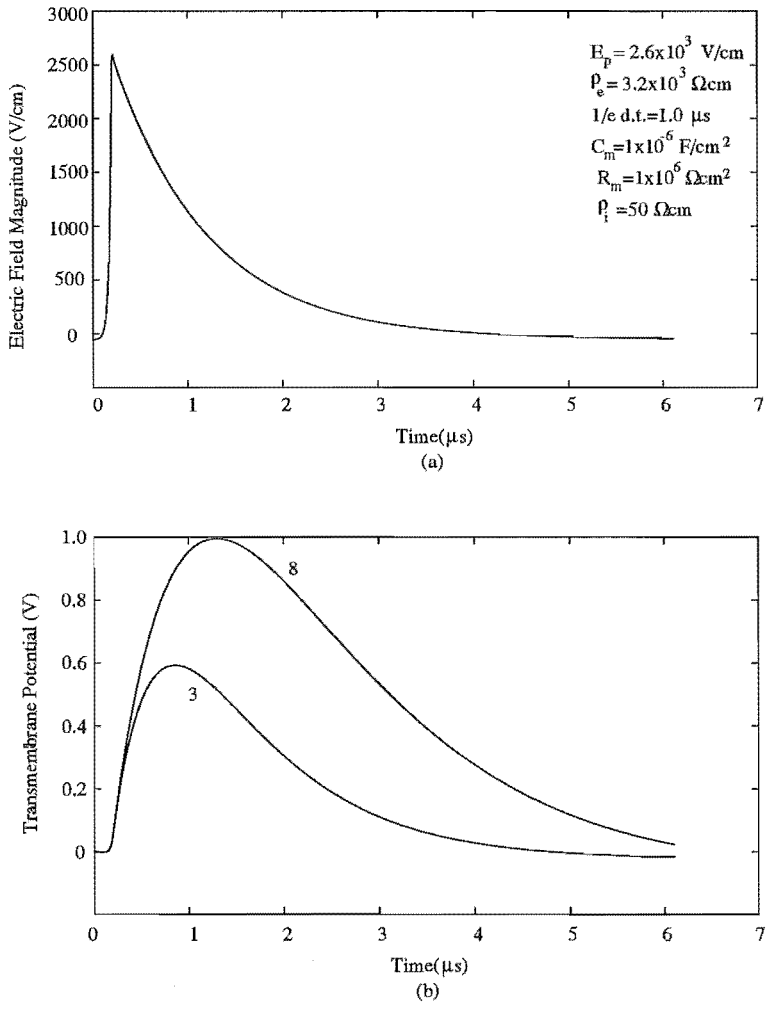


Figure 3.7 (a) 1μs $1/e$ decay time constant capacitive discharge impulse applied electric field. (b) Induced transmembrane potentials.

Figure 3.7 shows a capacitive discharge excitation field with a 1μs decay time and the induced transmembrane potentials. The peak potential of the 3μm cells is now 59% of the 8μm cells. The radius dependence on membrane potential is considerably reduced. This is a direct result of the membrane relaxation time T_r which, under

biological conditions, is also directly proportional to cell radius. However, to produce a transmembrane potential large enough for breakdown, the magnitude of the excitation field must be increased. Reducing the capacitive discharge impulse decay time to $0.1\mu s$ results in the $3\mu m$ cells membrane potential becoming 82% of the $8\mu m$ cells. The peak electric field has had to be increased to 13.3kV/cm. This is shown in Figure 3.8.

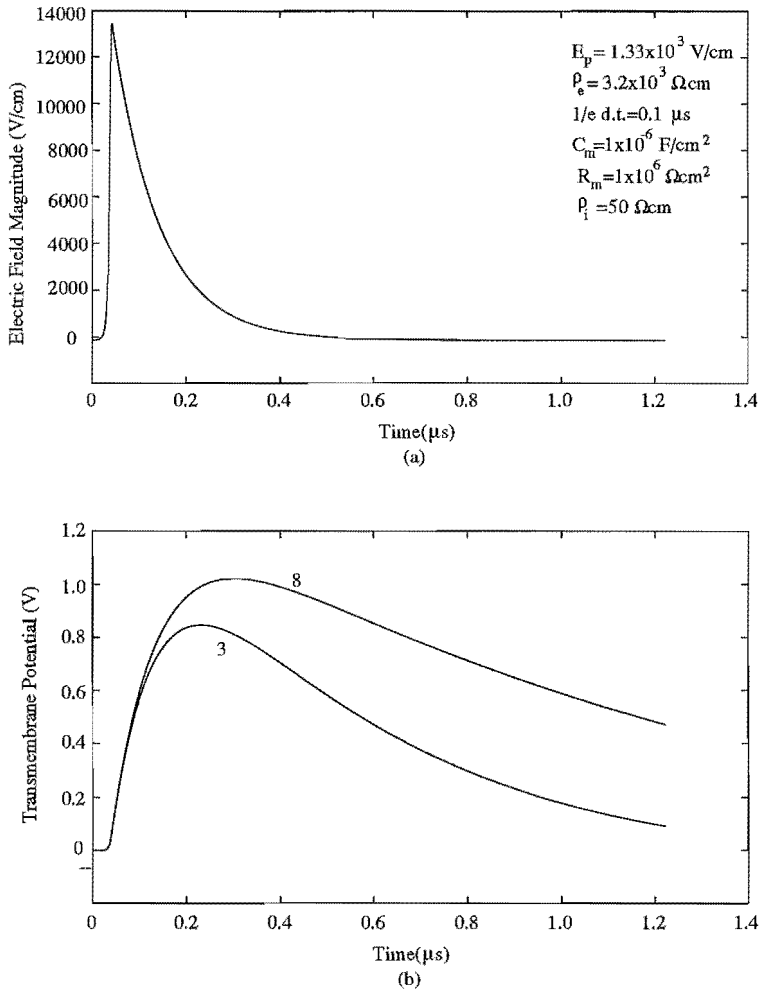


Figure 3.8 (a) $0.1\mu s$ $1/e$ decay time constant capacitive discharge impulse applied electric field. (b) Induced transmembrane potentials.

Physically, it becomes difficult to reliably produce impulses less than $0.1\mu s$ in duration. Therefore, if closer transmembrane potentials are required, T_r must be increased. Looking at Equation 2.2, the easiest way to increase T_r is to increase the external suspension medium resistivity ρ_e. If ρ_e is increased to $10^4\Omega$cm, then for a capacitive discharge of $0.1\mu s$ the percentage becomes 91%. Increasing T_r slows down the charge build up so yet higher electric field magnitudes are required. This is shown in Figure 3.9.

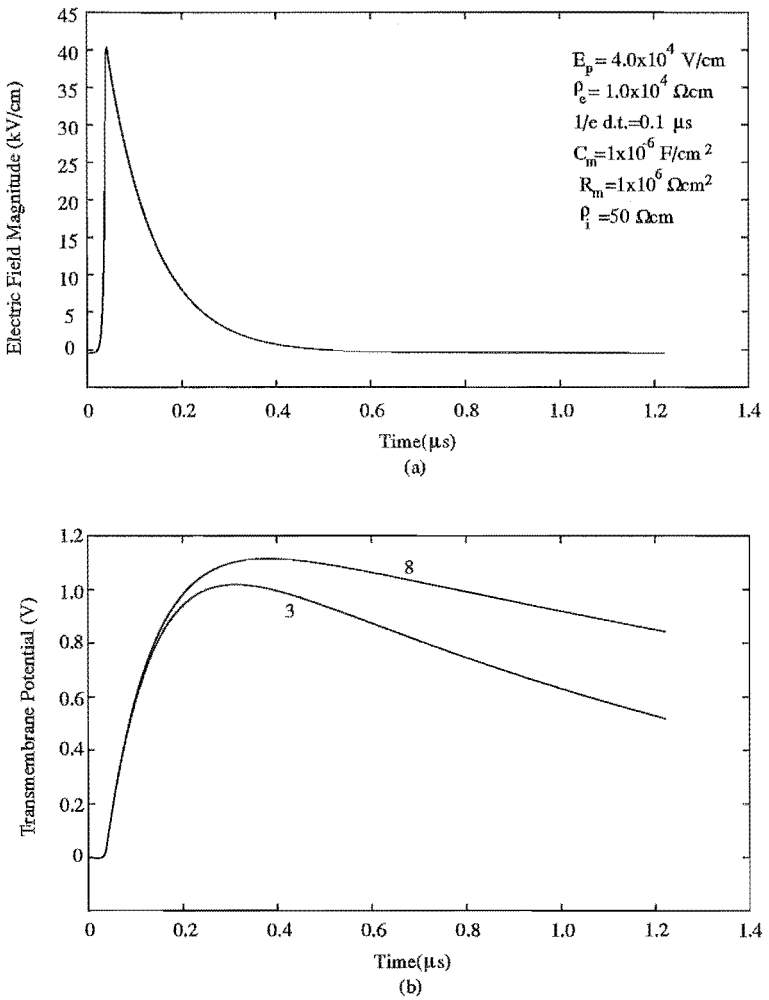


Figure 3.9 (a) 0.1μs $1/e$ decay time constant capacitive discharge impulse applied electric field. (b) Induced transmembrane potentials for an increased ρ_e.

There is however a practical limiting factor to how much ρ_e can be increased. Equation 2.4, Equation 2.5 and Equation 3.1 to Equation 3.4 have an extra product term known as the conductivity factor, $F(\sigma)$ [Neumann 1989]. $F(\sigma)$ is defined as,

$$F(\sigma) = \frac{\sigma_e \sigma_i (2d/a)}{(2\sigma_e + \sigma_i)\sigma_m + (2d/a)(\sigma_e - \sigma_m)(\sigma_i - \sigma_m)} \tag{3.5}$$

where σ_i is the cell or model interior conductivity, σ_m is the membrane conductivity, σ_e is the external suspension medium conductivity and d is the membrane thickness. In virtually all instances, σ_i and σ_e are much larger than σ_m. In this case Equation 3.5 can be approximated by,

$$F(\sigma) = \frac{1}{1 + \sigma_m \left(\frac{2+(\sigma_i/\sigma_e)}{2\sigma_i d/a} \right)} \tag{3.6}$$

If σ_m is smaller than about 10^{-3}S/cm, then $F(\sigma)$ is approximately equal to 1. This is the case for most practical electroporation and electrofusion applications, which is why $F(\sigma)$ is usually omitted from system equations. If σ_i is much greater than σ_e and σ_m, then Equation 3.5 can be approximated by,

$$F(\sigma) = \frac{\sigma_e \sigma_i (2d/a)}{\sigma_i \sigma_m + 2d/a(\sigma_e \sigma_i - \sigma_m \sigma_i)} \tag{3.7}$$

At the point where $\sigma_e = \sigma_m$, $F(\sigma) = 2d/a$. Thus, $U_m(t)$ will be reduced by a factor of about 10^{-3}. Therefore, σ_e should be kept more than two orders of magnitude larger than σ_m so that $F(\sigma)$ remains approximately equal to 1. Previous investigators have tried to overcome the radius dependence of transmembrane potentials by using a d.c. shifted sinusoidal radio frequency pulse which has a 100kHz frequency [Chang 1989]. But as Figure 3.10 shows, the difference between the transmembrane potentials still increases for the chosen radii. However, if the d.c. component is removed and the peak electric field maintained, the transmembrane potentials are brought closer together as shown in Figure 3.11. This suggests that a.c. electric fields symmetrical about a zero potential axis can reduce the radius dependence of transmembrane potentials more effectively than d.c. pulses with higher frequency components. Figure 3.12 shows the effect of using an 800kHz sinusoidal excitation field on transmembrane potentials. The 3μm cells have practically the same transmembrane potential as the 8μm cells after about 1 cycle. The peak field magnitude for the sinusoid is considerably lower than the impulse of Figure 3.9 which has a greater radius dependence.

3.3.3 Electrofusion Dynamics

The dynamics involved with membrane fusion initiated by electropermeabilization are still relatively unknown. The initial stages of electropermeabilization mimic those of electroporation and can be described by Equation 2.6. When membranes that have

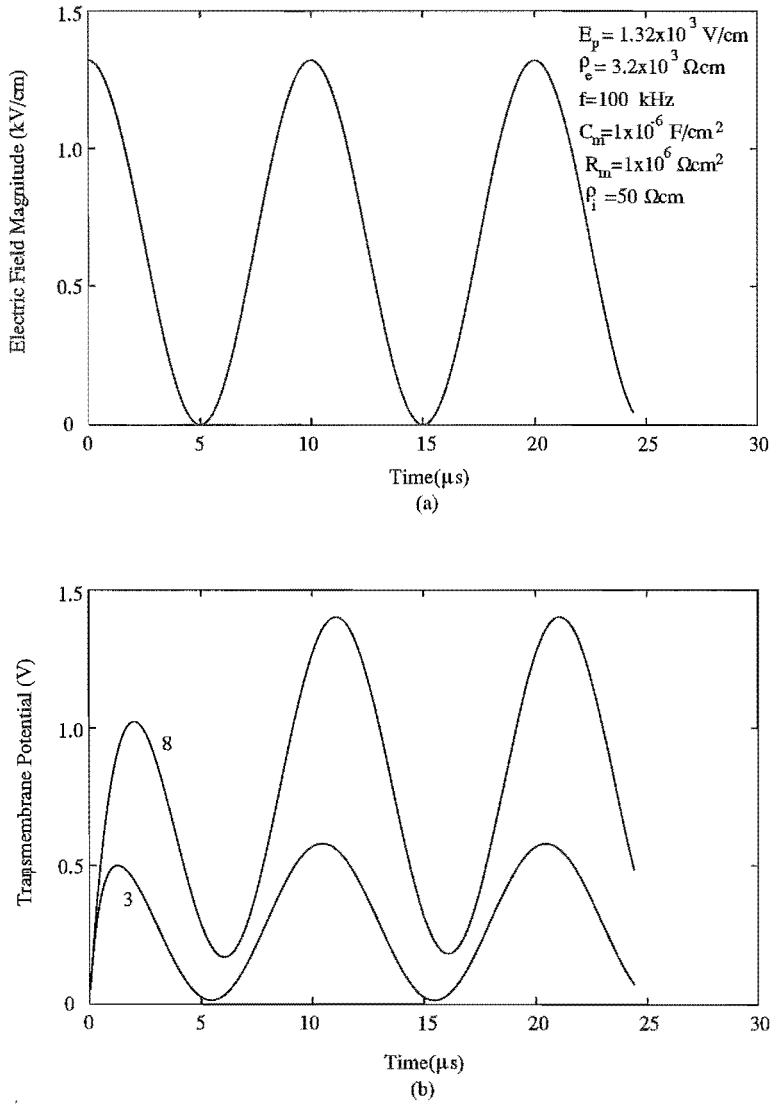


Figure 3.10 (a) 100kHz d.c. shifted pulse applied electric field. (b) Induced transmembrane potentials.

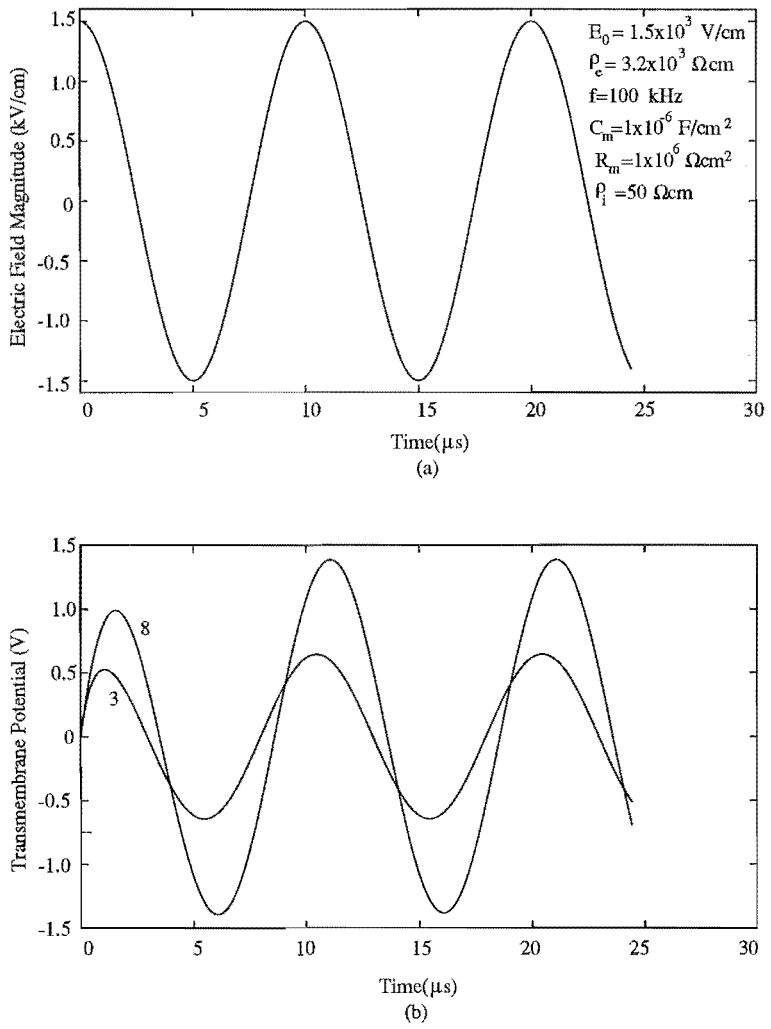


Figure 3.11 (a) 100kHz symmetrical sinusoidal pulse applied electric field. (b) Induced transmembrane potentials.

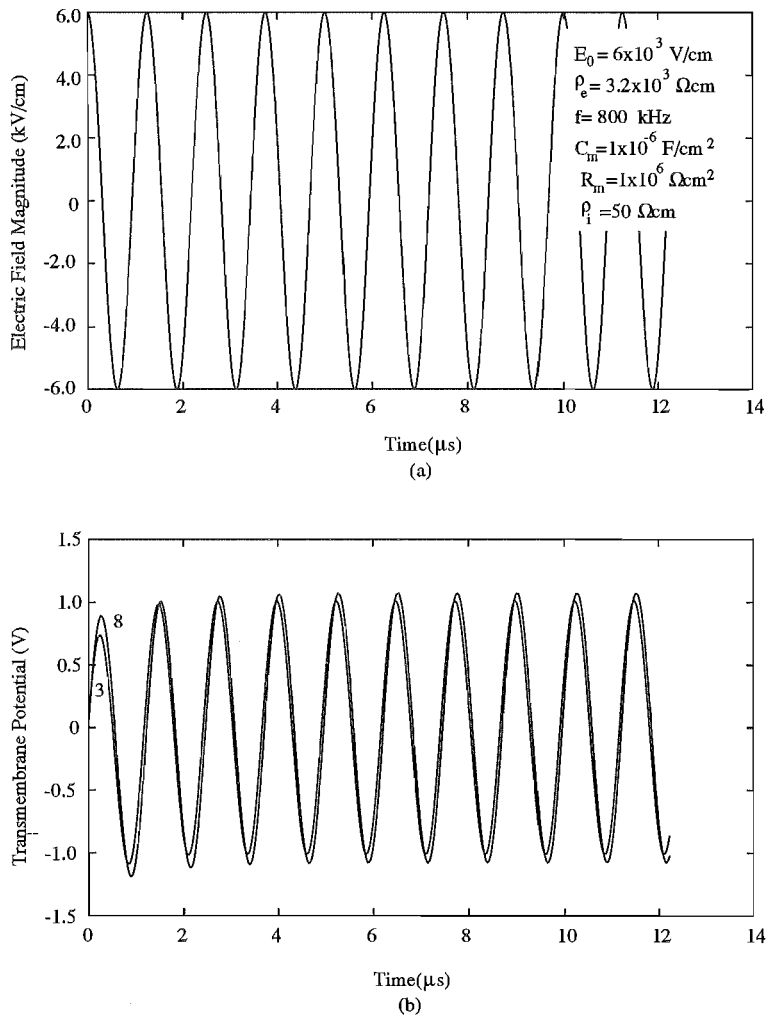


Figure 3.12 (a) 800kHz symmetrical sinusoidal pulse applied electric field. (b) Induced transmembrane potentials.

undergone electropermeabilization are in close enough contact, structural rearrangements begin to form membrane-membrane bonds between the cells [Dimitrov 1993]. The bond construct tends to increase the fusion zone. Bonds expand and merge until a steady state is achieved. Three final or steady states usually exist [Chernomordik and Sowers 1991]. In one, much of the cytoskelletal network remains intact so that the fused cells have an hourglass shape and the cytoplasmic constituents do not mix. A hybridoma so formed is not stable as the nuclei can not fuse [Bertsche *et al.* 1988, Zimmermann *et al.* 1990]. Another final state takes on a spherical morphology yet allows no cytoplasmic mixing. Once again, hybridomas with this form are unstable since the nuclei cannot fuse. The third steady state takes on a spherical form and allows cytoplasmic mixing. This then allows the nuclei to contact and possibly fuse which result in stable hybridomas.

If any of the cell fusion partners experience irreversible membrane breakdown, then that cell will lyse and no hybridoma will be produced.

Due to the different mechanisms involved, permeabilization pulses with low magnitudes and long durations, lower electrofusion efficiency. Therefore, for longer application requirements, the long pulses are split up into shorter pulse trains with recovery times between each pulse [Neumann *et al.* 1989]

3.4 BALLOON MODEL APPLIED TO ELECTROFUSION

The same balloon model introduced in Chapter 2 was used to investigate whether the numerical model predictions are accurate with respect to eliminating radius dependence of membrane breakdown by varying the d.c. impulse application time and/or varying the suspension medium conductivity [Gaynor and Bodger 1994a]. Higher frequency a.c. effects could not be investigated as the equipment required to generate the high voltage and frequency signals was unavailable.

3.4.1 Experimental Setup

A latex rubber spheroid was filled with domestic water, with a resistivity of 100Ωm, until the desired radius was reached. The balloon model was then suspended in a polyethylene tank filled with water. The tank water or external suspension medium was supplied by the same domestic water source mixed with quantities of deionised water. An insulated suspension rod was required to maintain the balloon in a static position.

The tank diameter was 800mm. Two 340mm by 480mm parallel plate electrodes spaced 500mm apart were placed in the tank such that the balloon model occupied a space midway between the electrodes. The tank diameter was sufficiently large so that any electric field established between the electrodes was relatively uniform and

not significantly affected by the tank sides. One of the electrodes was connected to a ground plane while the other was connected to the third stage output of a 14 stage, variable 1.4MV, inverted Marx impulse generator. A capacitive discharge impulse from the generator was then applied to the electrode, tank and balloon model. The experimental setup is illustrated in Figure 3.13.

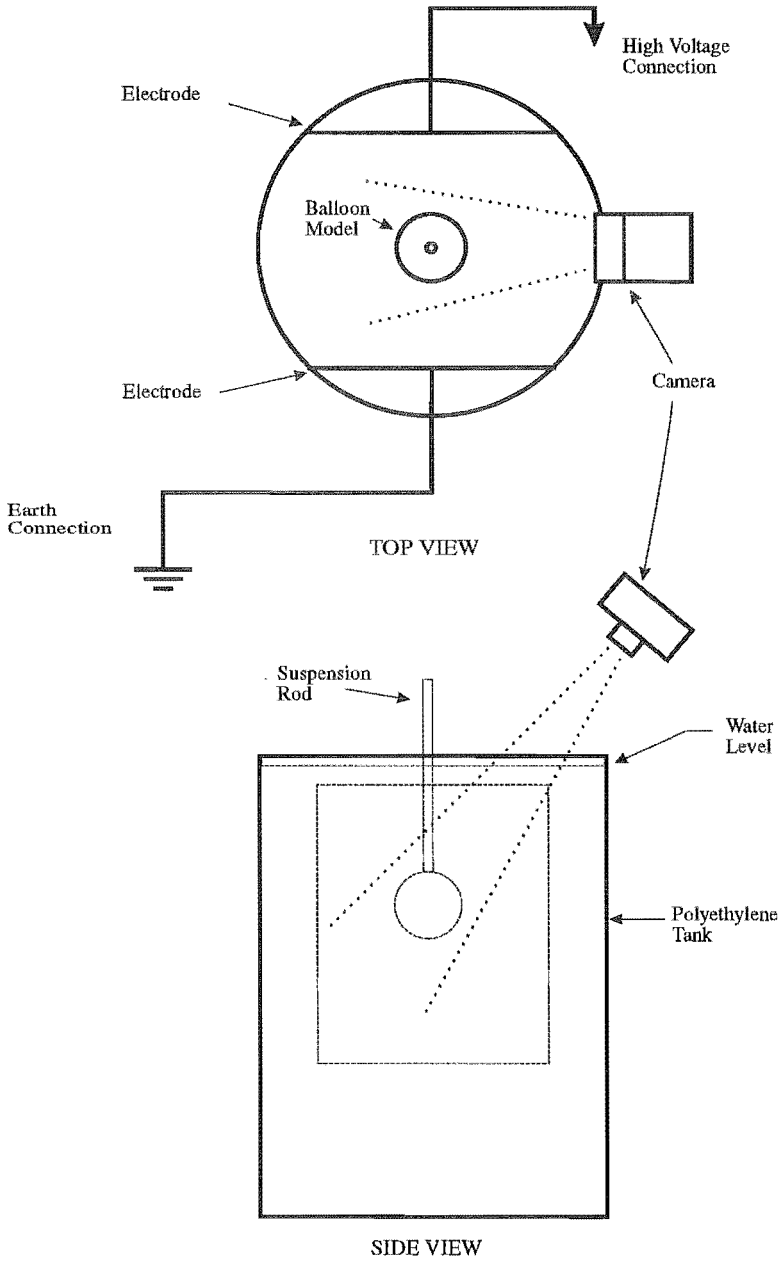


Figure 3.13 Balloon model experimental setup.

3.4.2 Experimental Method

Two specific balloon radii were used throughout the experimentation. They were 65mm and 80mm. These sizes were chosen as previous investigations showed that this range would allow membrane breakdown to occur at applied field magnitudes achievable by the apparatus available [Gaynor and Bodger 1994c].

Threshold dielectric breakdown of the membrane was qualified and quantified by photographic capture of membrane arcing and water droplet formation on the dried balloon surface. The camera position is shown in Figure 3.13.

The suspension water resistivity was varied by adding different quantities of deionised water to the tank water. The range of water resistivity was between 80Ωm and 5kΩm.

The decay time of the capacitive discharge from the impulse generator could be varied without altering the suspension medium resistivity by attaching a variable parallel resistor to the generator output.

All recorded peak electric fields and $1/e$ decay time constants have a maximum error of ±5%.

3.4.3 Experimental Results

The first test involved the 65mm radius balloon model and 90Ωm tank water resistivity. It was determined that the membrane breakdown threshold occurs at a peak applied electric field magnitude of 196kV/m and a $1/e$ decay time constant of 8μs.

The second test involved the 80mm radius balloon model and 90Ωm tank water resistivity. It was determined that the membrane breakdown threshold occurs at a peak applied electric field magnitude of 147kV/m and a $1/e$ decay time constant of 8μs.

The ratio of small and large model radii was 1.23. The respective inverse ratio of the peak applied electric field magnitudes required for threshold membrane breakdown was 1.33. This indicates that membrane breakdown threshold is close to the inverse of the radius. These results suggest that the conditions are adequately described by the steady state level of Equation 2.5 even though a capacitive discharge d.c. impulse is used. The reason for this is due to a small T_r [Gaynor and Bodger 1994a]. Thus charge carriers were able to appear across the membrane very and follow the excitation electric field. The peak electric field magnitude can then be used to calculate the transmembrane potential given by Equation 2.5.

T_r was then increased by increasing ρ_e from 90Ωm to 5kΩm. The membrane breakdown threshold of the 65mm balloon model increased to 198kV/cm with a $1/e$ decay time constant of 100μs. It was also found that the membrane breakdown threshold of

the 80mm balloon model had increased to 198kV/cm with a $1/e$ decay time constant of 100μs.

Within the accuracy of the measurements, no radius dependence on membrane breakdown is now observed. This indicates that $(\omega T_r)^2$ has been made more than an order of magnitude greater than 1. The $1/e$ decay time constant of the impulse has increased, which reduces the summed sinusoidal waveform frequencies. However, T_r appears to have increased to a greater extent.

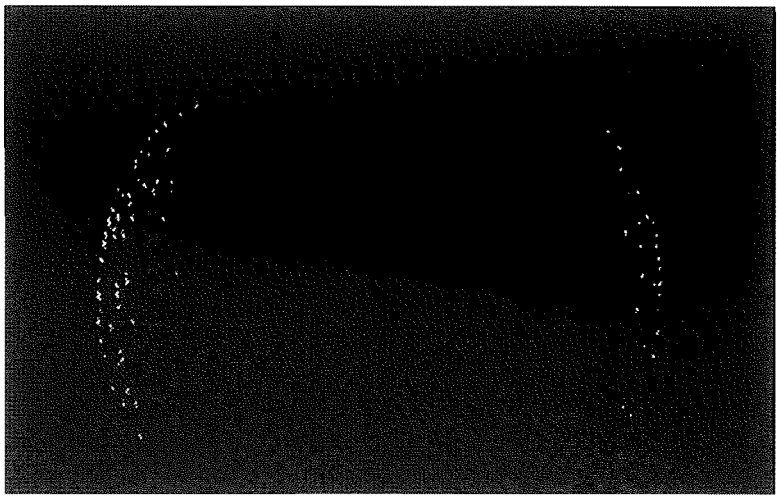
The external suspension medium resistivity was slowly reduced until a noticeable radius dependence of membrane breakdown was created. At a ρ_e of 111Ωm, the breakdown threshold was 198kV/cm and 180kV/cm for the 65mm and 80mm balloon models respectively. The $1/e$ decay time constant was measured as 12μs. By attaching a variable parallel resistor to the output of the impulse generator, the decay time constant of the applied electric field was reduced without altering ρ_e. When the $1/e$ decay time constant was reduced to 10μs, both balloon model sizes had a membrane breakdown threshold of 199kV/cm. Therefore, shortening the impulse decay time also reduces the radius dependence of membrane breakdown.

Figure 3.14(a) shows the typical photon emission caused by membrane dielectric breakdown in the model. Figure 3.14(b) shows the spatial placement of the balloon model from the fixed camera position. The breakdown arcing shown in Figure 3.14(a) indicated that asymmetrical breakdown has occurred by the unbalanced polar breakdown density.

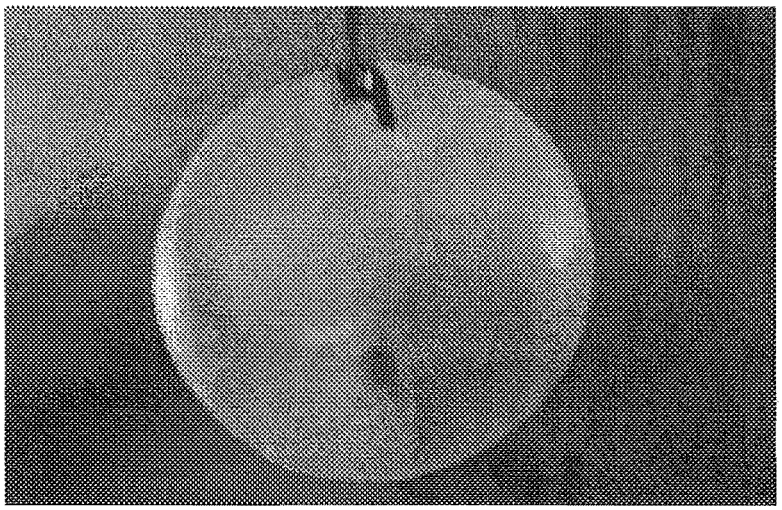
3.4.4 Electroporation Of Organelles Within Cells

In some electroporation applications it may be desirable to cause dielectric breakdown in the membranes of organelles including the nucleus membrane. Excitation electric field magnitudes that cause breakdown in the organelles would have to be used. These electric fields would generally lead to irreversible damage of the cell membrane and end in cellular lysis.

As an extension to the experimentation, a 65mm radius balloon model was placed inside a 80mm radius balloon model. Effectively this is much like a membrane bound nucleus inside a cell. When the resistivity of the tank water and both balloon model interiors was 90Ωm, the breakdown thresholds of the 80mm and 65mm balloon models were 147kV/cm and 196kV/cm respectively. The $1/e$ decay time constant was 8μs. When the resistivity of the tank water was 5kΩm and the resistivity of the internal water of both balloon models was 90Ωm, the threshold membrane dielectric breakdown of both balloon models was observed at a peak applied electric field magnitude of 198kV/cm. The $1/e$ decay time constant was 100μs. The photonic emission caused by membrane arcing during breakdown is shown in Figure 3.15.



(a)



(b)

Figure 3.14 (a) Typical experimental photon emission and (b) balloon model placement.

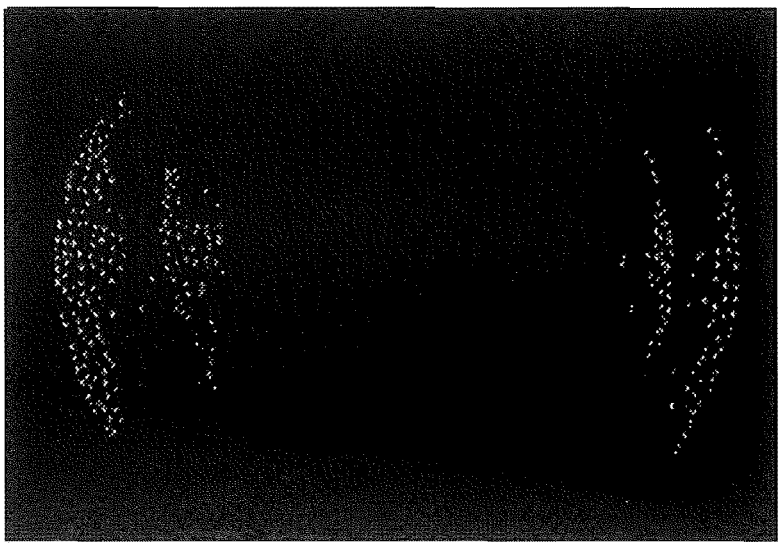


Figure 3.15 Photon emission from the breakdown of a balloon model within another balloon model. The internal resistivities of both models were 90Ωm and the suspension water resistivity was 5kΩm.

3.5 BIOLOGICAL ELECTROFUSION EXPERIMENTATION

Actual biological electrofusion tests were performed so that some of the positive aspects indicated by the models could be tested. Owing to equipment limitations, only the effect of shortening impulse application times on hybrid yields could be covered.

3.5.1 Electrofusion Apparatus

Electrofusion apparatus normally consist of four main sub-systems. An a.c. signal generator is required to produce the a.c. electric fields used in dielectrophoresis. A high voltage impulsē generator is required to produce the high magnitude electric fields which cause permeabilisation of the cell membranes. Some sort of cuvette/electrode arrangement is needed to hold the cell suspension which will, through its geometry, expose the cells to the electric fields produced. Finally, in order to isolate the a.c. signal generator from the cuvette/electrode arrangement during the application of the high voltage impulse, a suitable switching device must be incorporated into the apparatus. The electrical apparatus used for all recorded biological electrofusion experiments essentially consists of the four sub-units in a refined form.

The a.c. generator produces square waves between 300kHz and 1.5MHz with a maximum peak to peak voltage of 30V. It is capable of driving loads down to about 10Ω.

The d.c. impulse generator produces capacitive discharge impulses with a voltage range between 50V and 1500V and a $1/e$ time constant range between 0.1μs and 10^3ms. The impuse generator is also capable of driving loads down to 10Ω.

The cuvette/electrode arrangement consists of two 10mm x 25mm aluminium parallel plate electrodes with a separation of 2mm. The rest of the cuvette is constructed of clear acrylic. The cuvette size allows a maximum of 400μl of cell suspension to be loaded for each electrofusion test.

A switching device is used which not only isolates the a.c. signal generator when the d.c. impulse generator is ready to fire, but can also be set to determine the application time of the a.c. alignment signal to the electrodes prior to and after the application of the impulse.

All of these sub-systems are depicted in Figure 3.16.

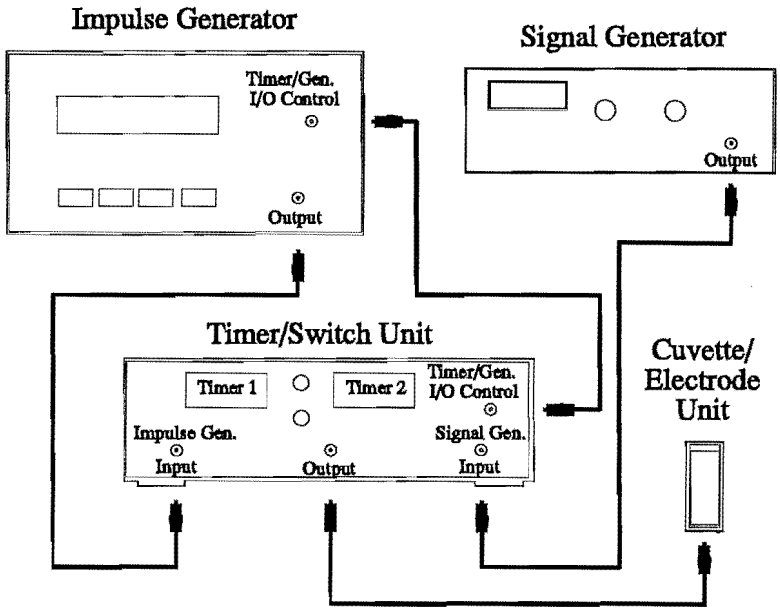


Figure 3.16 Experimental electrofusion apparatus setup.

3.5.2 Experimental Procedure

FOX-NY murine myeloma cells are grown in RPMI medium to densities that still allow log growth. RBF/DN mice are peritoneally (through the gut) injected with 150μl of 1mg/ml Progesterone-3CarboxyMethylOxine-Bovine Serum Albumin solution in Frends Complete Adjuvant, once a month for three months. Three days before harvesting the spleen for activated B cells, a mouse is peritoneally injected with 200μl of 1mg/ml Progesterone-3CarboxyMethylOxine-Bovine Serum Albumin solution in Phosphate Buffered Saline. Suspensions in RPMI with a 1:1 ratio of a known number of lymphocytes and FOX-NY cells are then made. Aliquots of the suspension are spun down at 2000rpm for 10min. The supernatent is removed and the cells resuspended in a hypo-osmolar fusion medium to a concentration of 6×10^6 cells/ml. At this con-

centration the cells are in close enough proximity to allow pearl chain formation in a uniform applied electric field. The fusion medium is a solution of 1mM Calcium ions, 1mM Magnesium ions and 0.2M glucose in sterile double distilled water. The fusion medium has a pH of 7.5. 400μl samples of the suspension are transferred to individual cuvettes. Dielectrophoretic alignment of the cells is achieved with a 100V/cm, 1MHz square wave applied for 45s. The electropermeabilization pulse or pulses follow within 30ms of removal of the alignment electric field. The alignment field is reapplied about 20ms after the pulse for 20s. The 20ms delay is quite important as this allows the impulse rotated cells to return to their original orientation before close contact is again induced. All samples are electrofused within 15mins of being suspended in the fusion medium as this increases fusion yields [Foung *et al.* 1990]. The treated cells are left to incubate and form stable fusion sites for 10 min before 3ml of phenol red free RPMI is added. It has been found that using phenol red free growth medium for about 24 hours after electrofusion can increase hybridoma yields by 50 times [Foung *et al.* 1990]. Equal volumes of the suspension are tranferred into 12 wells of a 96 well tissue culture plate. Hybrid clone cultures are counted after 7 days incubation at 37^0C with 5% CO_2.

3.5.3 Experimental Results

Effects on fusion efficiency of two different $1/e$ decay time constants were investigated. One decay time constant was set at 12μs and the other was set at 5.5μs. Only two time parameters were studied as biological resources and apparatus variability were limited.

The results were normalised for each experiment to give a percentage difference in highest hybridoma yields. This was done in order to minimise the observed fluctuations in fusion yields due to the effects of physical and biological variations between individual experiments. The percentages were then averaged over all the experiments. The results showed that the 12μs $1/e$ decay time constant tests generated, on average, about 20% fewer hybridomas than the 5.5μs tests. The variance of the data is, however, very high (about +25 and -40%) owing to the relatively small statistical set. Further experiments are required to reduce this variance and determine the actual difference in efficiencies.

It was also observed that the peak efficiencies were achieved between about 3.2-4.0kV/cm for the 5.5μs tests and between about 2.2-3.0kV/cm for the 12μs tests. This follows the theoretical model trends and balloon model observations on the increasing peak electric fields required to induce membrane breakdown for shorter application time impulses.

3.6 A.C. PULSE ELECTROFUSION/ELECTROPORATION SYSTEM

Technically it should be possible to design and construct very effective a.c. electrofusion/electroporation systems and apparatus. The type of system that is developed

for particular applications will be determined by two main factors, cost and desired efficiency. In this section, a low cost yet functional design is first presented. A second design is then considered which is a research tool used in the determination of optimal electrofusion/electroporation procedures.

Assuming dielectrophoresis will be utilised to induce close cell-cell contact, then a source of a.c. voltage that can generate electric fields in the 10^2V/cm range with a variable frequency between about 10kHz to 2MHz is required. Potentially higher electric field magnitudes will be needed to induce electropermeabilization. Therefore, a source of a.c. voltage that can produce electric fields conventionally thought to cause electropermeabilization in bacteria should be used. The frequency range of the high voltage a.c. source should also be between about 10kHz to 2MHz.

3.6.1 A Simple A.C. Pulse System

In a simple electrofusion system, the dielectrophoresis electric field is applied to a cuvette, with parallel plate electrodes, for a set amount of time. It is then removed and the electropermeabilization pulse is applied. After the pulse or pulses, the dielectrophoresis field is again applied for a set amount of time. This procedure can be automated with a very simple timer/switch device.

One of the major expenses in electrofusion and electroporation apparatus is the high voltage pulse generation components. High voltage devices, due to their nature, are expensive. Low voltage devices are not. Therefore, generating the desired waveforms at a lower voltage and somehow amplifying the voltage to the required level, could be beneficial. Amplifying the voltage with some kind of semiconductor or valve device would defeat the cost saving precautions. Using a wide bandwidth transformer would be a better option.

With the advent of relatively cheap, wide bandwidth, high permeability ferrites, linear voltage transformations up to the low MHz region can be performed at a low cost. As such, a simple transformer could be used to generate the high voltage a.c. pulses from a considerably lower voltage. Also, since the dielectrophoresis field is in the same frequency range, the transformer could be used to enable construction of the dielectrophoresis appararus out of even lower voltage components. Inherent transformer characteristics produce voltage isolation between sets of windings linked by the same core. This allows a single core to link the output to the dielectrophoresis and high voltage pulse apparatus. A simple block circuit diagram of a potential system is shown in Figure 3.17. All of the elements shown can easily be built from readily available components at relatively low cost. The system is quite functional and high efficiency electrofusions or electroporations should be achievable. Simple modifications such as dynamic amplitude control of the dielectrophoresis signal may improve functionality. Basic microprocessor control can also be inexpensive [McCormick *et al.* 1992].

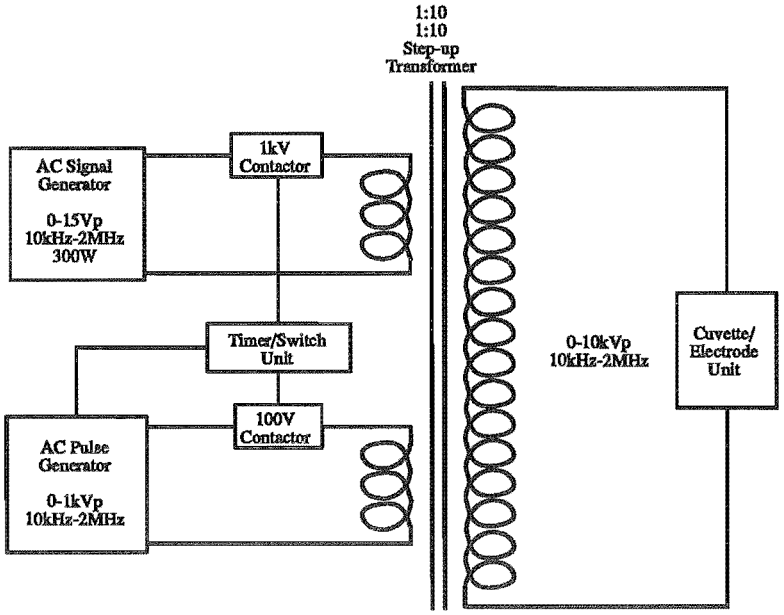


Figure 3.17 Block diagram of a possible low cost electrofusion/electroporation system.

3.6.2 An Optimisation Research Tool

In this situation, capital expenditure considerations are secondary to system versatility and variability. Design aspects may be based on actual physical parameters rather than approximations. A tool so developed will most likely determine optimal specifications. However, it will also contain a great deal of redundant variability.

Dielectrophoresis is still assumed to be used. It is efficient and easily implemented. Once again a source of a.c. voltage that can generate electric fields in the range of $10^2 - 10^3$ V/cm is required. The type and amplitude of the a.c. waveform should be dynamically variable, as a particular waveshape/amplitude combination may produce an optimum cell-cell attractive force. The frequency should be variable between about $10^2 - 10^7$Hz, to accomodate most of the frequencies that have been used for dielectrophoresis or cellular alignment. A more efficient means of producing close cell-cell contact may be developed in which case that method could replace dielectrophoresis in future systems.

The permeabilization pulse or pulses should have the same characteristics as described above. In addition, dynamic control of the pulse parameters such as amplitude and frequency should be incorporated. Pulse control could be based on variables such as suspension impedance which indicates the degree of electropermeabilization. This sort of control should eliminate the loss in electrofusion or electroporation efficiency owing to variation in biological parameters between experiments.

Transition characteristics from the dielectrophoresis field to the electropermeabi-

lization field and back should be variable. The transitions could be combinations of immediate step changes, smooth merging or spaced with dead zones. A block diagram

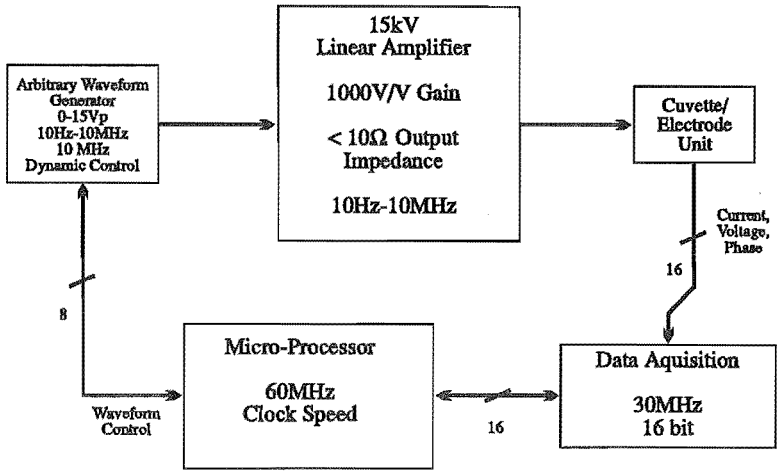


Figure 3.18 Block diagram of a possible electrofusion/electroporation optimisation research tool.

showing a system that could incorporate all the aspects mentioned is given in Figure 3.18. The apparatus required to realise this system are technically producable although very expensive.

3.7 DISCUSSION

It has been shown that, in many instances, application of multiple d.c. impulses increase fusion yields [Neumann *et al.* 1989, Chang *et al.* 1992b]. An a.c. system also exposes cells to multiple breakdown inducing electric fields. A sinusoidal excitation electric field can produce transmembrane potentials required for breakdown on positive and negative half cycles.

If a cell exposed to a high magnitude d.c. electric field impulse experiences asymmetrical breakdown and has an a.c. electric field applied immediately after the d.c. impulse, transient cellular rotation may occur [Zimmerman 1982]. A major requisite of electrofusion is that the cell membranes that are to be fused must be in close contact and in a state of high permeability. The d.c. impulse causes dielectric breakdown and a state of high permeability in the polar regions facing the electrodes. An a.c. electric field is usually applied to cell suspensions to induce close cell contact and may cause cellular rotation after asymmetrical breakdown has occured. Thus, the probability of fusion may be reduced. An a.c. pulse may reduce this rotation in producing symmetrical dielectric breakdown. Other investigators [Chang 1989, Chang *et al.* 1992a] have tried using sinusoidal pulses for electrofusion with promising results. However, the experimentation was limited by undesirable constraints. Frequencies above 400kHz, where

reduction in radius dependence becomes noticable for cells with significantly different radii, were not fully investigated. These experiments did indicate that another mechanism may be aiding membrane breakdown. This involves mechanical vibrations in the membrane brought about by the radio frequency nature of the electropermeabilization pulse. Reinforcement of this hypothesis is given from a bioelectrorheological electromechanical model that effectively predicts the observed experimental results [Pawlowski *et al.* 1993].

The numerical model shows that, for real biological parameters pertaining to a monoclonal antibody application, radius dependence of transmembrane potential is almost eliminated when a symmetrical 800kHz sinusoidal a.c. electric field pulse is used. This is an extreme case of the cell radii being very different. In many instances the cell sizes are much closer. A lower frequency and hence lower magnitude electric field could then sufficiently reduce the radius dependence. Electroporation of highly heterogeneous cell populations could also be improved by reducing radius dependence.

Comprehensive evidence of the beneficial effect of an a.c. electropermeabilization system has been shown [Tekle *et al.* 1991]. A bipolar or a.c. square wave pulse was used in an electroporation application. Fluorescence imaging showed that symmetrical breakdown was achieved. Qualitative data also showed that, cell viability was higher and more efficient electropermeabilization was achieved, compared to d.c. pulses.

A more comprehensive numerical model could incorporate the active components of dielectric breakdown. This would enable the model to determine optimal applied electric field durations for electrofusion or electroporation.

The material used for the balloon model is not of the nature that it can be fused by the electrofusion process. It does, however, possess similar high electric field effect characteristics as cellular membranes. Therefore, the model can be used to produce quantitative data of membrane breakdown aspects such as threshold levels, morphology and mechanisms.

Biological experimentation produces mainly qualitative data of membrane breakdown and fusion efficiency. The data is qualitative as observable biological function usually determines the results. Biological function is a secondary effect of membrane breakdown and might not be quantitatively indicative of the physical processes involved.

Increasing the suspension medium resistivity to eliminate the radius dependence of membrane breakdown must reduce $F(\sigma)$ to some degree. As the breakdown thresholds were not substantially larger for the 60mm radius balloon, the reduction in $F(\sigma)$ was negligible.

The model has shown that it is also possible to cause radius independent membrane breakdown for a balloon within a balloon. This configuration is indicative of an organelle such as a membrane bound nucleus within a cell.

Being able to make the nucleus of a cell highly permeable may be beneficial to electrofusion applications. It is a prerequisite that for a fused hybrid to be stable, the nuclei must also fuse [Foung *et al.* 1990]. Cell fusion partners are chosen that will readily undergo spontaneous nuclei fusion. This limits the types of cells that may be fused. Generating the same kind of electropermeabilization in the nucleus membrane as experienced by the cell membrane may aid in initiating nuclei fusion. This could increase the range of cells that can be fused by increasing the hybrid compatibility of cell fusion partners. Also, research involving the production of transgenic animals via electroporation [Barsoum 1990, Inoue *et al.* 1990] may have a purpose for the ability to make the nucleus membrane artificially permeable to genetic material. Drug loading of organelles within cells may also be accomplished.

Results from the biological electrofusions have shown that reducing the time constant of conventional capacitive discharge d.c. impulses produce higher fusion yields. It should therefore be possible for most users, with their existing apparatus, to obtain more efficient electrofusions. A drawback with eliminating the radius dependence of membrane breakdown, is that higher magnitude electric fields are required. Developing an a.c. pulse electrofusion system should reduce this problem. A.c. electric fields were shown, by the numerical model, to eliminate radius dependence and generate a sufficiently high transmembrane potential for dielectric breakdown at considerably lower peak magnitudes than d.c. impulses.

The amount of research on human monoclonal antibodies has been severely limited by the small number of activated B-lymphocytes that may be taken from an individual [Steenbakkers *et al.* 1993]. Also, direct contamination of a human with a particular antigen may be unethical and/or undesirable. It has been postulated that increasing hybrid yields only one order of magnitude higher than presently achievable will greatly increase the availability of human monoclonal antibodies [Foung *et al.* 1990]. It may be possible by realising and refining the propositions made in this chapter and optimising biological parameters, to achieve an order of magnitude increase in efficiency.

3.8 CONCLUSIONS

The results from this chapter have shown that the problem due to a radius dependence on transmembrane potential should be solvable. Direct physical experimentation using conventional d.c. impulses provided corroberation and validation to the models and their predictions. Also, problems due to excessive electric field magnitudes, cell rotation and asymmetrical breakdown should be reduced by the use of a.c. excitation electric fields.

Graphical representation of excitation fields and transmembrane potentials greatly enhances the ability to understand, design and control new electrofusion parameters. This can save time and expenditure in research projects involving electrofusion.

A balloon model has been presented that showed theoretical indications for cellular membrane breakdown behaviour are well founded. It has also provided some qualitative data that would be very difficult to obtain through biological experimentation. Qualitative data is essential for a more thorough understanding of any process whether it is physical, chemical or biological.

Technically, production of a.c. pulse based electrofusion and electroporation apparatus is quite feasible. The researcher has to decide on how much they are willing to spend on this equipment. Decisions will be based mainly on available capital expenditure and the degree of variability and versatility required for their applications.

The present and future applications of electrofusion are extremely important. Many of these applications require the most efficient systems possible. Therefore, electrofusion optimisation research has a challenging yet potentially very rewarding future.

Chapter 4

H.M.E.F. KILLING OF LIQUID BORNE BIOLOGICAL CONTAMINATES

4.1 INTRODUCTION

During investigations on electroporation and electrofusion it was observed that cell viability was severely reduced when pulsed electric fields were either too large, too long or both [Neumann *et al.* 1989]. This effect has possible ramifications in the killing of biological contaminates in liquids such as water and some hydrocarbons.

Over the years, as world populations have increased, demand for drinking water has correspondingly increased. Fresh ground and surface water supplies have always been a limited resource and population settlements are normally situated close to some kind of water supply. As a direct result of population demand and location, lower quality water has to be used and the available water is being chemically and biologically contaminated.

Developed countries have determined classifications of water quality which are dependent on concentrations of various chemical and biological pollutants [Pontius 1990]. A minimum drinking water quality is usually maintained which often requires substantial treatment of local water supplies. Third world countries also have badly polluted water supplies. However, the drinking water is often left untreated mainly due to capital costs and complexities of water treatment materials and plants.

It is the biological contamination of water which most often produces illnesses when consumed through drinking, particularly in developing countries where 40% of all deaths and 60% of all diseases are directly related to biologically contaminated drinking water [Filopure 1992]. The general seriousness of biological water pollution has resulted in the development of a number of disinfection methods. The type of water treatment used is determined by factors such as turbidity, ion content, mineral content, capital costs, running costs, biological diversity and efficiency. High magnitude electric field (h.m.e.f.) killing of these contaminates could be an alternative method of treatment.

Hydrocarbon fuels such as diesel and kerosene (aviation fuel) are often infected with

fungal and bacterial bodies which reside in tiny droplets of water [Sheridan *et al.* 1972]. The cells attach to the interior of fuel systems where they may produce corrosive compounds that attack the fuel system materials. Eventually the fuel system integrity can be compromised. This is a particularly serious problem for aeronautical situations where the fuel tanks also serve as wings. Clogging of filters and fuel injection systems can also occur when mats of fungi dislodge from fuel system surfaces [Miller and King 1985].

Some techniques have been developed to deal with this problem. They are usually based on micro-filtering and adding detergents or biocides to fuel, although there is also a device that uses permanent magnets [De Bug. 1990]. In some instances, h.m.e.f. effects may provide a solution to the problem.

Other liquids could also be treated using high magnitude electric fields. Fruit juice, milk, syrup, sauce and cosmetics are all possible candidates. Each liquid requires individual consideration owing to the different substances and applications involved.

In any situation, h.m.e.f. lysing effects must provide some sort of advantage over currently available techniques. It is thus necessary to determine the limitations of h.m.e.f. killing as this will indicate the application bounds and areas of usefulness.

4.2 H.M.E.F. WATER DISINFECTION

A biological cell suspended in water can have a large transmembrane potential induced by the application of a h.m.e.f. in an identical manner to electroporation and electrofusion. Beyond a particular electric field magnitude and application time, micropores in the cell membrane will be of the size and density that cause irreversible breakdown and cell lysis. By this means, biologically contaminated water supplies can be disinfected. It is important to note that disinfection implies killing of most, but not all organisms. The term for total biological destruction is sterilisation.

4.2.1 Primary Disinfection Design Specifications

There are a number of fundamental specifications for the proposed disinfection process that must be catered for. These are,

1. The h.m.e.f. must somehow be applied to the entire water volume.
2. Electrical properties of the water to be treated must allow the generation of the physical processes that induce cell lysis.
3. The process must reduce expected biological contaminates to acceptable levels.
4. Some method of water transport to remove treated water and supply untreated water.

5. The technology used in the process should be economically and practically feasible to the application.

6. Water quality can not be chemically or physically degraded to unacceptable levels.

These specifications are so fundamental, that water could be replaced by any prospective liquid that might be treated.

4.2.2 Basic Electrode/Chamber Design

In order to apply the required electric field to the water, parallel bar electrodes were made to be in contact with the water. To satisfy the rest of primary specification 1 and a solution to primary specification 4, an insulating material (acrylic) was fashioned which forced a continuous water flow between the parallel electrodes. The basic electrode and chamber system is shown in Figure 4.1.

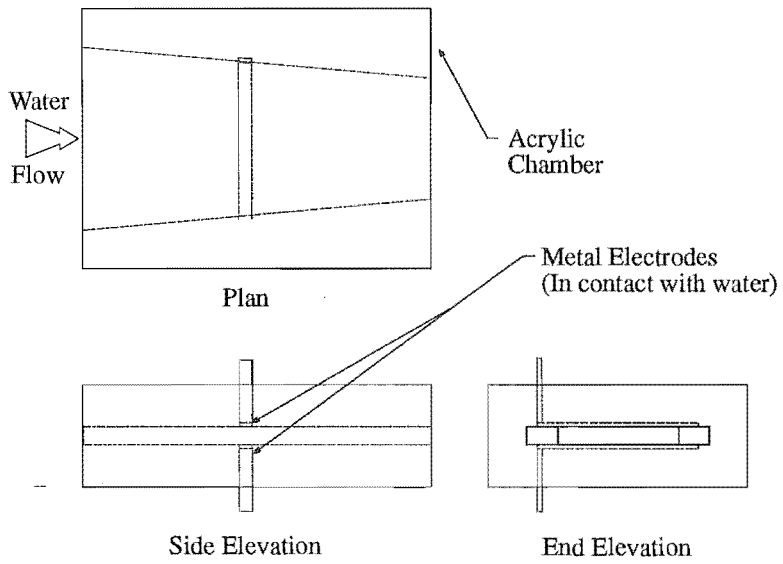


Figure 4.1 Basic electrode and chamber system.

4.2.3 Basic Electrical Properties Required Of Water

In order for the physical processes of pore formation to occur, the water to be treated must be relatively conducting with respect to the cell membrane. For most naturally occurring water sources, this criterion is met. However, if the water is distilled or deionised, the conductivity could be reduced to a level whereby excessively high applied electric fields would be required to induce breakdown transmembrane potentials. Practical limitations on conductivity will be discussed in Section 4.5.

4.2.4 Basic Electric Field Composition

The option of continuous water flow produces particular constraints on the applied electric field. In order to satisfy primary specification 1, the applied electric field (and hence voltage across the electrodes) must apparently be continuously present with respect to the water flow. Both continuous d.c. and a.c. fields can be used under the chosen water flow parameters. Pulsed fields, with delays significant to the flow rate through the parallel electrodes, may result in some volumes of water being insufficiently treated.

4.3 EXPERIMENTAL INVESTIGATIONS

To see if primary specifications 3, 5 and 6 can be accomplished by the h.m.e.f. method, experimental studies were carried out. Various parameters such as electrode dimensions, spacing and materials, water flow rate and conductivity, electric field composition, magnitude and generation, and biological contaminates, were investigated to determine their effects.

4.3.1 Previous Investigations

A masters project [Jaquiery 1992] ascertained many of the parameter effects and a provisional patent was filed as a result. The following is a compressed presentation of the information gained.

It was found that the time any particular cell spent between the electrodes in the presence of an appropriate electric field, greatly affected the killing efficiency. This is directly related to the electric field application time lysing effect observed in electroporation and electrofusion. That is, the longer a cell is exposed to a breakdown inducing electric field, the greater the probability of lysing. Also, as the electric field magnitude increases, the time spent under the electrodes to obtain a particular lysing rate, reduces.

Water conductivity was reduced, with a mixed bed deionising resin, to between 1-10μS/cm for all experimentation to reduce power requirements.

Direct current and 50Hz sinusoidal a.c. electric fields were used to discover their effects on kill rate efficiency. Direct current proved to be more efficient at killing than the a.c. waveform for a particular rms electric field. This was attributed to 'dead band' regions in the a.c. waveform where the electric field was too low to induce membrane breakdown effects. Figure 4.2 illustrates these regions. The result is operatively the same as a pulsed electric field with significant delay times.

Various electrode materials were considered and the two most suited metals in an affordable range were titanium and stainless steel. These metals have relatively large dissociation potentials and thus do not suffer so much from electrolysis [Fried 1973].

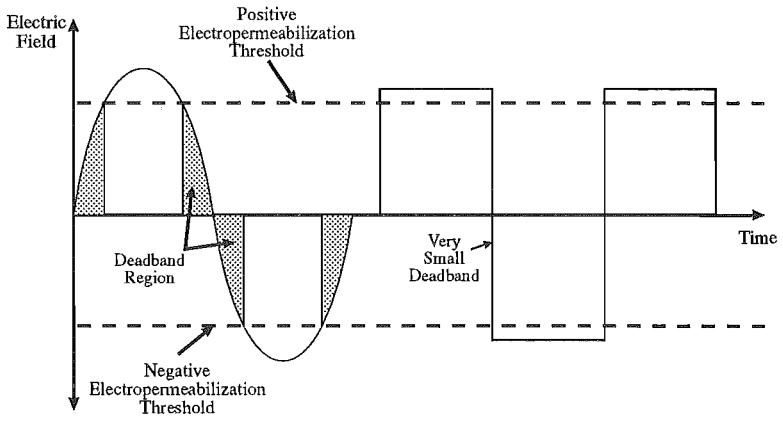


Figure 4.2 Dead band regions associated with a.c. waveform zero magnitude crossings.

Titanium is also extremely biocompatible so that any titanium injected into the water will have no adverse effects if consumed.

Two different cell types, serratia marcescens and bacillus megaterium, were tested which simulated extreme conditions that might be encountered. Serratia marcescens is a very small, gram negative bacterium that has a radius smaller than 0.5μm [Grimont and Kreig 1981]. As the potential developed across a cell membrane is directly proportional to the cell radius, then smaller cells require higher magnitude electric fields to kill them. Bacillus megaterium (gram positive) were tested as this bacterium can become an endospore under adverse conditions such as residing in water for a length of time. Endospores have very thick and tough outer shells which are different to bilayer lipid membranes [Madigan and Brock 1988]. These endospores are then likely to behave differently to h.m.e.fields. An electric field magnitude of 20kV/cm applied to serratia marcescens cells for an average time of 2ms, results in about 93% lysing. The same field magnitude and application time produced about 24% lysing in the bacillus megaterium endospores.

Since the water conductivity was reduced to between 1-10μS/cm, electrolysis effects were minimal. Electrolysis is dependent on current density, so for a given electrode surface area, increasing the current increases electrolysis. Reducing the conductivity of the water reduces the current drawn due to an applied electric field. This also reduces the amount of electrolysis.

Two aspects which remained unresolved by the previous work, were the effects of higher water conductivity and reduction of the dead band regions in a.c. waveforms.

4.3.2 Effects Of Increasing Water Conductivity And Decreasing A.C. Waveform Dead Bands

To check the effects of increased water conductivity, raw domestic water with a conductivity of about 120μS/cm was passed through a titanium electrode/chamber system that had previously been used with low conductivity water. An applied d.c. electric field of only one fifth the magnitude (4kV/cm) previously generated, resulted in the initiation of arcing between the parallel electrodes. Below this magnitude, fine bubbles were observed to be appearing between the electrodes. Also, the power consumption had increased by over one order of magnitude. Substantial corrosion of the positive electrode only was observed after less than five minutes of operation.

It was noticed that when a 50Hz sinusoidal electric field was applied, an electric field of 5kV/cm could be attained before arcing occurred. Severe electrolysis was still observed, although now on both electrodes.

In addition to being an effect of current density, electrolysis is also an effect of time. Therefore, if a higher frequency a.c. waveform is used, less electrolysis should occur. A quasi-square wave, variable frequency voltage generator was constructed in order to determine the consequence of frequency. It was verified that increasing frequency resulted in lower electrolysis effects for the same rms current density. Figure 4.3 shows electrolysis and oxidation effects of three electrodes at various frequencies from 50Hz to 1kHz, at an rms current density of 0.5 A/cm^2. The quasi-square wave also reduced the dead band regions associated with a.c. waveform zero crossings. This is illustrated in Figure 4.2.

When the dead band regions are reduced, the operatively equivalent pulsed a.c. waveforms have reduced delay times. The duty cycle of breakdown bands to dead bands increases which should result in an increased killing efficiency.

It was noticed that the increased power consumption led to significant heating of the water. The amount of temperature rise is affected by the flow rate which is in turn, constrained by minimum application times. This suggests that if the water conductivity is too high, boiling point temperatures may be reached.

4.4 DISINFECTION OF HYDROCARBON LIQUIDS

It was stated in Section 4.2.1 that the primary specifications were fundamental to all liquids to be treated by the h.m.e.f. process. Herein lies an apparent major problem. Primary specification 2 states that the electrical properties of the fluid to be treated must allow the generation of the physical processes that induce cell lysis. However, the non-conducting nature of hydrocarbons does not facilitate the induction of transmembrane potentials. The reason for this, is that the cells and the tiny water droplets they are in are much more conductive than the hydrocarbon liquid. In the presence of an

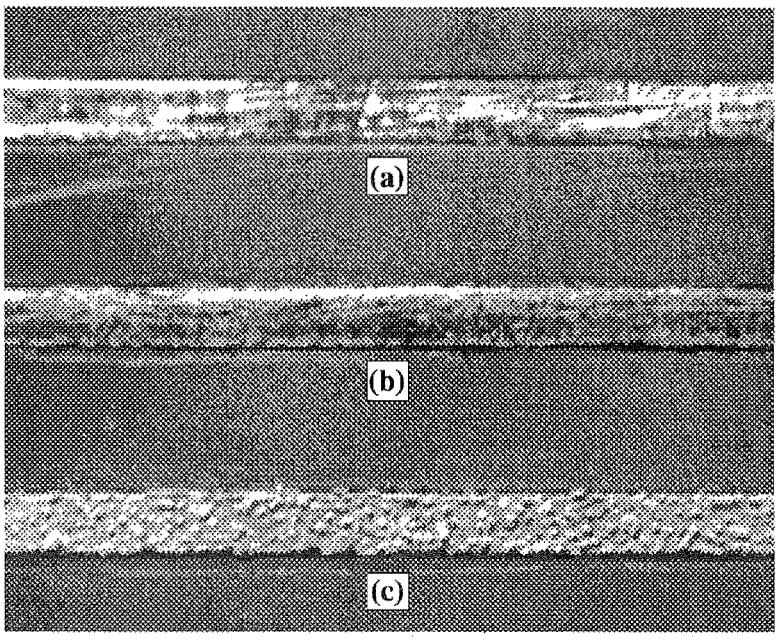


Figure 4.3 Electrolysis and oxidation effects on titanium metal electrodes after 5min operation at 0.5 A/cm^2 for (a) 1kHz (b) 500Hz and (c) 50Hz applied square wave electric fields.

electric field, the water droplets and cells form equipotential (zero electric field) zones in the bulk liquid. The electrode/chamber system designed for the treatment of water is then probably of no use in the treatment of hydrocarbons as it stands.

Although the arguments above appear conclusive, experiments were carried out that tried to lyse E-coli cells suspended in kerosene with the electrode/chamber style and electric fields used in the water experiments. Results indicated that little or no lysing occurred as expected. Re-evaluation of the system was necessary.

4.4.1 Modified Electrode/Chamber Design

If electrode separations were reduced to slightly smaller dimensions than the water droplets, then in order for the droplets to pass through the electrodes, they must contact both electrode surfaces. As soon as both electrodes are contacted by the water droplets, an electric field is induced across them and primary specification 2 is satisfied.

Water droplet sizes can be as small as 10μm in diameter which sets the maximum allowable electrode spacing. To facilitate a reasonable flow rate, a grating geometry could be arranged as shown in Figure 4.4. Significantly lower voltages would generate the required electric fields. As a consequence, lower voltage and lower cost componentry can be used for the field generation.

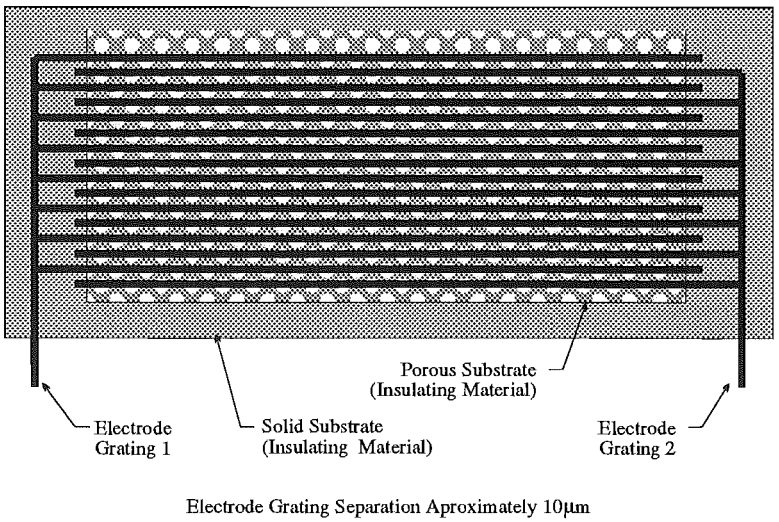


Figure 4.4 Possible micro-grating electrode configuration for hydrocarbon and other very low conductivity liquids. Fluid flow is perpendicular to the plane of the page.

4.4.2 Pre-Treatment Of Hydrocarbon Liquids With Charge Carriers

If the hydrocarbons could in some way be doped with conducting material without altering their functional properties, it might be possible to continue using the basic electrode/chamber system developed for water disinfection.

In some instances, detergents and biocides are added to kerosene and diesel. It is then conceivable that some chemical could be found to raise the conductivity of the hydrocarbon liquids. Experimental work on water disinfection indicates that a conductivity of close to 1μS/cm would be sufficient to allow induction of the transmembrane potentials required for irreversible breakdown.

4.5 DISCUSSION

When any conducting solid is placed in a liquid that contains charge carriers and an electric field is applied, electrolysis occurs when a certain threshold current density is passed. The threshold current density is a function of the electrode material. It is also a function of frequency. This indicates that it should be possible to reduce or eliminate electrolysis effects if a high enough frequency a.c. waveform is used. Unfortunately, there are factors which complicate and limit the degree of a.c. frequency range.

For a practical square wave a.c. signal, there will always be a certain transition time from one potential to the other. As the frequency of the waveform increases, the transition time becomes a larger percentage of the total signal. Thus, as the frequency increases, the duty cycle of killing electric field bands to dead bands decreases. Before this problem is likely to occur, another detrimental effect will probably be

experienced. This is the effect of lowered transmembrane potentials caused by high frequency shorting of the membrane capacitance. Frequencies above about 100kHz result in significant reductions in transmembrane potentials for any particular applied electric field. Therefore, the maximum useful frequency will be below about 100kHz.

Producing power square wave a.c. signals requires a considerable amount of sophisticated circuitry. Primary specification 5 could be compromised in situations where construction of the circuitry can not be accomplished due to lack of funds or unavailability of componentry. This may only be a possibility in developing countries.

In a practical application, it will be desirable to use the lowest practicable frequency. The reason for this is that the best duty cycle is encountered at the lowest frequency. However, there is a correlation between the minimum frequency that can be used with a particular liquid conductivity and the maximum allowed electrolysis effect. This sets the minimum practical frequency.

From the experimentation performed on the disinfection of water, it is apparent that liquid conductivities down to about 1μS/cm still provide enough charge carriers to allow the induction of effective transmembrane potentials. Be that as it may, results from electroporation and electrofusion studies imply that this is probably the lower limit. Any lower conductivities will probably result in significant lowering of transmembrane potentials for a given applied electric field.

4.5.1 Water Disinfection

Experimentation on the higher conductivity water showed the generation of bubbles between the electrodes. It has been suggested that this is due to hydrolysis of the water which forms gaseous hydrogen and oxygen [Jaquiery 1992]. This is unlikely to be the major component of the bubbles as the collected gas did not appear to be flammable. It is more probable that the power being delivered to the water is causing dissolved gas to come out of solution. Whatever the source, the bubbles cause substantial nonlinearities in the electric field which significantly lowers the apparent dielectric strength of the water. This is the main reason for the initiation of arcing at lower electric field magnitudes. Higher frequency electric fields resulted in less bubble evolution and higher apparent dielectric strengths. Degassing the water before treatment may reduce this problem.

Owing to the power being dissipated in the higher conductivity water, a considerable temperature rise is developed. Boiling of the water could be induced if the source water temperature and the temperature rise add to reach boiling levels. Since boiling also produces bubbles, this is an undesirable state to create. The overall result of this is that there is a maximum liquid conductivity limit that is directly dependent on the source liquid temperature. Also, at elevated temperatures, gases are more likely to come out of solution.

The water treatment system based on the h.m.e.f. process does have some important advantages over other water treatment systems.

Water treatment systems are generally quite complex, expensive and sensitive to certain variations in water conditions [J. M. Montgomery Consulting Engineers 1985]. The h.m.e.f. system is relatively simple, cheap and can be designed to be robust to variations in water conditions.

Many water disinfection systems degrade the water quality by the addition of chemicals or the generation of reactive compounds from inert compounds in the water [Pontius 1990]. With the choice of an appropriate electrode material in the h.m.e.f. system, water quality can be essentially unaffected.

4.5.2 Hydrocarbon Liquid Disinfection

The disinfection of hydrocarbon liquids by h.m.e.f. systems required certain modifications in order for primary specification 2 to be satisfied. If, as suggested, electrode separations are reduced to close to 10μm, then certain other physical aspects must be considered.

The hydrocarbon liquid must be pre-filtered to remove particulate matter larger than 10μm. Since most fuels are already filtered, this is probably of little concern.

Accurately separating electrodes by 10μm along lengths measured in centimetres and keeping them there will require reasonably sophisticated technology. This may make designing and building prototypes difficult, although large scale production should be relatively straight forward.

Having only 10μm electrode separation will result in an increased fluid flow impedance. If the viscosity and/or surface tension of the liquid is too large then the pressures required to force the liquid through the electrode/chamber system to obtain a particular flow rate, may be excessively high. The crossectional area of the electrode/chamber system could be increased until the desired flow rate was attained for a given liquid pressure. This may, however, result in an unrealistic set of dimensions.

Other physical aspects of hydrocarbons are extremely favourable to the h.m.e.f. system. The very low inherent conductivity of the liquids (about 20 nS/cm) would permit the use of d.c. electric fields as no bulk electrolysis would occur. The only electrolysis developed would be at the points where water droplets span the electrode separation. Electrode corrosion would be minimal and periodic reversal of electrode polarity would effectively double the lifetime of the entire electrode system. The low liquid conductivity and close electrode separation would permit the use of low voltage, low power and low cost devices. This may balance the extra cost of fabricating the modified electrode/chamber system.

Conversely, adding compounds to increase the liquid conductivity would facilitate the use of the simple electrode/chamber system, but require higher voltage, power and

priced circuitry. Whichever option is chosen will be determined by experimentation, prototyping and application results.

4.5.3 Disinfection Of Other Liquids

As long as all the primary specifications can be met, just about any fluid could be treated by a h.m.e.f. system. It is unlikely that the highly conductive liquids such as fruit juice and milk could be more effectively treated than presently existing methods. Overcoming electrolysis and power consumption effects would simply make h.m.e.f. treatment unfeasible. Less conductive liquids offer better possibilities.

Disinfecting low conductivity liquids by h.m.e.f. systems will not alter their physical or chemical makeup. This is a distinct advantage where preservatives and other additives are either undesirable or not permitted such as in many foodstuffs and hypoallergenic cosmetics.

4.6 CONCLUSIONS

It has been shown that h.m.e.f. disinfection of liquids can be achieved simply and cheaply, particularly if certain physical conditions are encountered. Low (but not exceptionally so) conductivity liquids provide the best opportunities for the process although this is not essential.

The simplicity and available robustness of h.m.e.f. systems could provide an alternative water disinfection process that developing countries are able to use, or where domestic applications are warranted.

The increasing demands on available water sources has forced people to treat lower quality water. By far the most common method of water disinfection is by the addition of chemicals. The result has been a loss of water aesthetics, reduction in the chemical quality of water and development of chemically resistant organisms. The h.m.e.f. process does not work by chemical but physical action which has no effects on water aesthetics and does not reduce its chemical quality.

Hydrocarbon liquids should be able to be effectively disinfected through an appropriate h.m.e.f. treatment system. The low power consumption and cost of the process offer significant advantages over current disinfection techniques. However, the actual structure of a practical h.m.e.f. system is still to be determined.

As in all disinfection techniques, the h.m.e.f. process has definite limitations. Electrolysis and power consumption can in some instances make the process completely unfeasible. Thus it is unlikely that h.m.e.f. treatment of highly conductive liquids will ever be practical.

Whatever the liquid that may be treated, h.m.e.f. disinfection does not require or produce environmentally or socially undesirable chemicals. This is a distinct advantage

over most other disinfection systems currently available and should be considered as a factor more important than cost.

Chapter 5

COMBINED D.C. AND IONIC INDUCTION OF DEDIFFERENTIATION

5.1 INTRODUCTION

It has long been known that low magnitude electromagnetic fields are present throughout a biological system. They exist in the most primitive cell to the most complicated organism. Prevalent amongst these electromagnetic fields are d.c. electric fields. It is well known that d.c. electric fields are present in all nerve function and also normally appear across cell membranes [Hoppe *et al.* 1983]. It has been observed that application of external electric fields of specific magnitudes and spatial direction can directly affect the function of nerve cells [Plonsey and Barr 1988] and the inherent cellular transmembrane potential [Neumann *et al.* 1989, Chang *et al.* 1992b]. These effects have been used in both therapeutic and research applications.

Less commonly known is that low level d.c. electric fields are present at most wound or growth sites [Becker 1990, Smith and Best 1989]. Application of similar magnitude d.c. electric fields to these sites have been shown to produce marked effects [Liboff and Rinaldi 1974]. Some of these effects have been linked to the process of cellular dedifferentiation which is a key component of tissue regeneration and many cancers.

Dedifferentiation is the process in which mature, specialised cells return to a less mature state. During dedifferentiation, genes that code for other cell types can be made available for use by effectively de-repressing them. Some cancers are formed by an external stimulus causing the expression of an inappropriate gene, called an oncogene, without a large change in the DNA of the cell [Ogawa 1993]. It may be possible to treat these cancers by turning off the oncogene through an induced dedifferentiation/redifferentiation process.

A possible means of artificially inducing this process involves the use of metal ions accompanied by a very low d.c. current. The silver ion appears to be the most effective [Becker 1990]. This chapter explores the effect of combined d.c. and metal ion action on some specific cancer cells in an attempt to observe aspects of the dedifferentiation process. Elements of a possible melanoma treatment are also discussed.

Melanoma is fast becoming one of the more commonly contracted cancers in caucasians. This has been attributed to increasing solar ultraviolet radiation resulting from a thinning of the ozone layer and Western society fashion aesthetics pressuring fair skinned people to aquire tans. After prognosis of a potentially malignant melanoma site in the skin, a large section of tissue is removed from this region. Obvious and permanent scarring is produced as a result. It may be possible to devise a superficially invasive non-scarring technique to induce dedifferentiation of the melanoma cells to a level whereby the body's natural mechanisms can re-differentiate the cells to a normal state.

5.2 CELL MICROBIOLOGY

The field of cellular micro- and moloecular biology incorporates a vast amount of theory and practice [Pelczar *et al.* 1986, Atlas 1984, Scott 1988, Freifelder 1983]. Therefore, only the topics relevant to the matter under investigation will be introduced.

5.2.1 Differentiation

Life of most multicellular organisms begins with the fertilisation of a female gamete (ovum) by a male gamete (sperm) to produce a zygote. This single cell contains all the genetic information necessary to form a complete individual and is the most immature cell possible. For the first few divisions, every cell is as totipotent as the original zygote. This means that if any of these cells were placed in an appropriate environment, they could produce a wholly complete organism [Larrick and Burck 1991].

From the embryo, through numerous divisions, the complete life form develops [Curtis and Barnes 1989a]. The process in which a cell transforms from a simple embryonic type to a mature, specialised type in the adult is called differentiation. This involves restricting, or repressing, all genes specific to other cell types and is known as differential regulation of gene expression [Larrick and Burck 1991]. Upon cell division, the resulting cells have either kept their same maturity or have matured slightly. When the cell matures some aspects of its genetic code are repressed and the resulting cells become more specialized [Goodenough 1984].

To form any complete individual, many different cell types are required. As a higher organism develops, the cells form three rudimentary tissue types - the endoderm, which develops into the glands and intestines; the mesoderm, which becomes the muscle, bones and circulatory system; and the ectoderm, which becomes the skin, sense organs, and the nervous system. At these levels, differential regulation of the genes in the cells is apparent. As the cells differentiate into mature tissues, only specific sets of genes remain active in each kind as portrayed in Figure 5.1(a). In general, whilst the genes are being repressed, each cell still contains an identical copy of the original DNA [Curtis and Barnes 1989a]. However, each set of cells can make only certain types of messenger

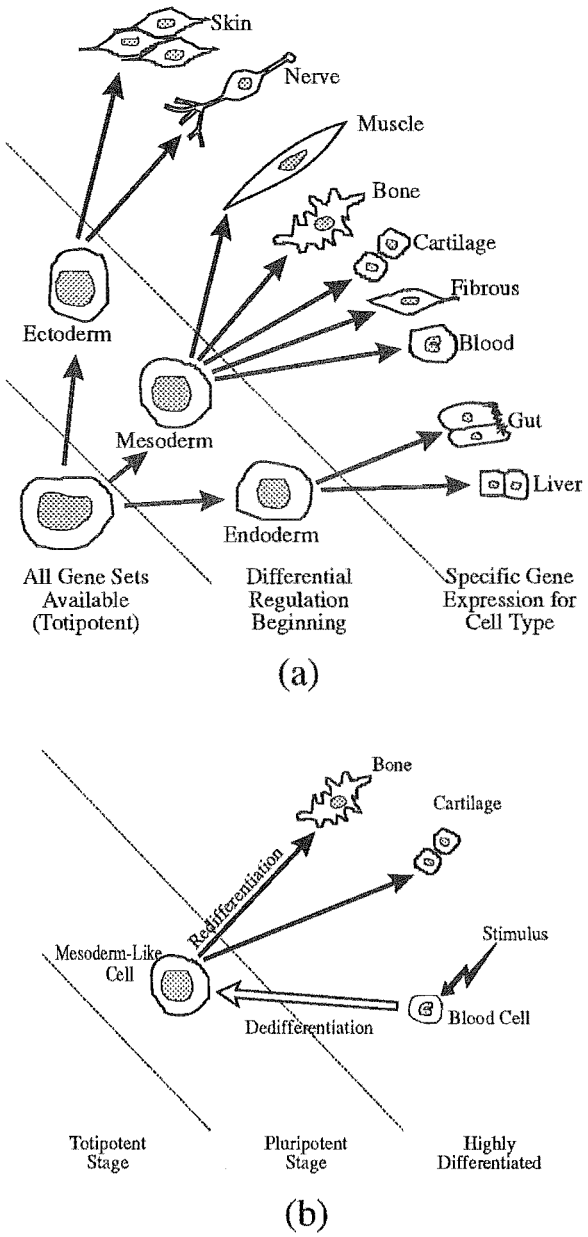


Figure 5.1 (a) Simplified differentiation sequence for mammalian cells. (b) Possible dedifferentiation/redifferentiation path.

ribonucleic acid (mRNA) which instructs the ribosomes to make the particular proteins that distinguish one cell from another.

The differentiation process continues until cells have reached their full maturity. They are then at their most specialized form or terminally differentiated. A mature cell assumes a characteristic shape that enables it to function effectively in the tissue that it belongs to. A fully differentiated cell often ceases to undergo any further cell division [Wessles and Hopson 1988].

5.2.2 D.C. Electric Fields And Currents During Differentiation And Growth Of Cells And Tissue

Owing to the ionic content of cells and tissue, when an electric field exists so does an electric current. Being mutually inclusive, the effects of one are neccessarily the effects of the other.

It has been postulated that asymmetrical distribution of ion pumps within a cell membrane can induce an effective transcellular d.c. electric current and hence electric field [Hoppe *et al.* 1983]. This electric field may affect asymmetrical ion pump distribution in neighbouring cells thus generating tissue level electric fields. Electric fields of this type have been found preceeding and during local differentiation and growth in all plants and animals. It is generally acepted that the measured electric fields are not simply by-products of metabolic activity in differentiation or growth. They are an essential component without which these functions can not proceed. Interfering with the electric fields has resulted in cessation of differentiation and alteration of the organisms structural morphology.

5.2.3 Dedifferentiation

Certain conditions can affect cells in such a way that the process of specialization (differentiation) is reversed to some degree. This is naturally called dedifferentiation. Once thought not possible (that cells could not 'de-mature'), studies in different areas such as cancer [Yano 1993, Kieback *et al.* 1993] and regeneration [Smith 1988, Becker 1990] research have shown this not to be the case. However, attempts to understand the dedifferentiation mechanism or to control the process have been difficult [Maatta 1993, Agata 1993, Becker 1990].

An example in selective dedifferentiation involved the ability of the salamander to convert cancer tissue into normal tissue [Becker 1990]. Cancer cells from frogs were induced to form a tumor in the limbs of salamanders. If the limbs were amputated through the tumor, they would regenerate normally eliminating all signs of cancer. Biopsies showed that the cancer cells which contained the frog DNA in their nuclei were now normal muscle and bone tissue. This could only be accomplished through a

dedifferentiation/re-differentiation process. If the tumors were left to grow, the cancer eventually fatally consumed the salamanders.

Another example of selective dedifferentiation was revealed when researching the mechanism by which bone fractures heal in frogs [Becker 1970]. It was found that red blood cells, which still contain a nucleus in amphibians, that had clotted in the fracture were turning into bone cells. This indicated that the blood cells were dedifferentiating into primitive pluripotent cells and then redifferentiating into bone cells as shown in Figure 5.1(b). It was theorised that the red blood cells were caused to dedifferentiate, not by chemical action, but rather by the application of a minute amount of electrical current. This current was probably generated by the mechanism described in Section 5.2.2. In mammals it was found [Becker 1985a] that by running a current of similar magnitudes through a bone fracture that had refused to heal using conventional methods, the bones were knitted together. Apparently the bone marrow had dedifferentiated and then turned into bone tissue.

If enough pluripotent or multipotent cells are created at a site of injury, eg. an amputated limb, the body may be able to regenerate the missing part. An electric current due to injury flows down the perineural cells (the cells surrounding the nerve fibres) and back through the injury site. This current is negative in animals that can regenerate spontaneously, such as the salamander, and positive in animals that can not. By changing the direction of the current flow in non-regenerates, regeneration can be initiated [Becker 1990, Liboff and Rinaldi 1974]. In mammals it was found that bone regenerates in this way.

Other mammalian cell types needed an extra stimulus to induce dedifferentiation. This stimulation was found to be a metal ion such as silver [Becker 1990, Becker 1985b]. Silver possesses many qualities which may be useful in medicine and other areas [Becker 1990, Marino *et al.* 1974, Doyle 1989, McLean *et al.* 1993]. One of these is silver's disinfection effect upon microorganism contaminates in solutions, for example, in water supplies [Hamilton Miller *et al.* 1993]. Another major aspect of silver is it's low level of toxicity for humans [Albert 1988]. This is unlike most other metals: copper, aluminium, lead, and mercury are all considered extremely toxic to the human bio-system [Chang 1984].

Silver, like most metals, gives off positive ions when placed in a solution. The rate of ion diffusion into the solution is greatly increased when a current is passed between electrodes [Gooding and Frank 1989, Fried 1973]. The current causes metal ions to diffuse from the positive electrode into the solution. The number of ions that diffuse into a solution through the electrodes can be controlled by the current magnitude.

5.2.4 Cancer

Cancer cells are often highly specialized cells which have lost some of their differentiation [Albert 1988]. They have regressed to a simpler state or dedifferentiated to some degree. They divide continuously though inefficiently. The division can be much slower than that for normal cells, though usually a greater percentage are in division at any one time. This can lead to a very high cancer growth rate in comparison to normal cells, most of which are terminally differentiated and do not divide frequently or at all [Albert 1988, Wessles and Hopson 1988].

Cancer is the collective term for at least two hundred diseases characterised by unrestrained self cellular growth. They are categorized into two main groups [Wessles and Hopson 1988]. One group involves Tumours. These are solid masses of cancerous cells which can occur anywhere on the outer and inner surfaces of the body, (Carcinomas), or in any soft tissue, (Sarcomas). The other group involves Leukaemias (Leucomas), and Lymphomas. These arise in the blood forming cells of the bone marrow and lymph nodes.

It has been shown that some forms of cancer are caused by the uncharacteristic expression of a particular gene known as an oncogene [Larrick and Burck 1991]. This is usually caused by an external stimulus, such as carcinogenic chemicals or some form of radiation. A cell's normal growth regulation is disrupted and this leads to runaway mitosis. It is hypothesised that Melanoma is this kind of cancer, caused by UV radiation.

As previously stated, it was thought possible to reverse the gene expression in melanoma by dedifferentiating the cells using d.c. and silver ion action, and then let the body redifferentiate them back into normal skin cells [Marino *et al.* 1974]. The first stage of this ongoing investigation, involved conducting in-vitro experiments .

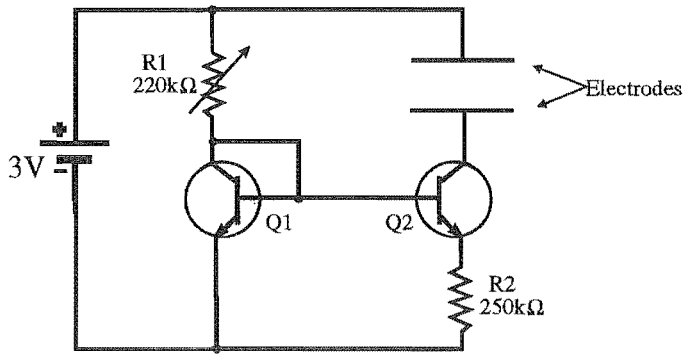
5.3 EXPERIMENTAL MATERIALS AND METHODS

The equipment used for the in-vitro tests can be separated into two main components. The first is a very low level constant d.c. current source which can generate currents in the range observed at regeneration or wound sites [Hoppe *et al.* 1983, Liboff and Rinaldi 1974, Smith and Best 1989, Becker 1990]. The second is an electrode/culture dish arrangement that exposes the cells under test to the d.c. current (or electric field) and a supply of metal ions.

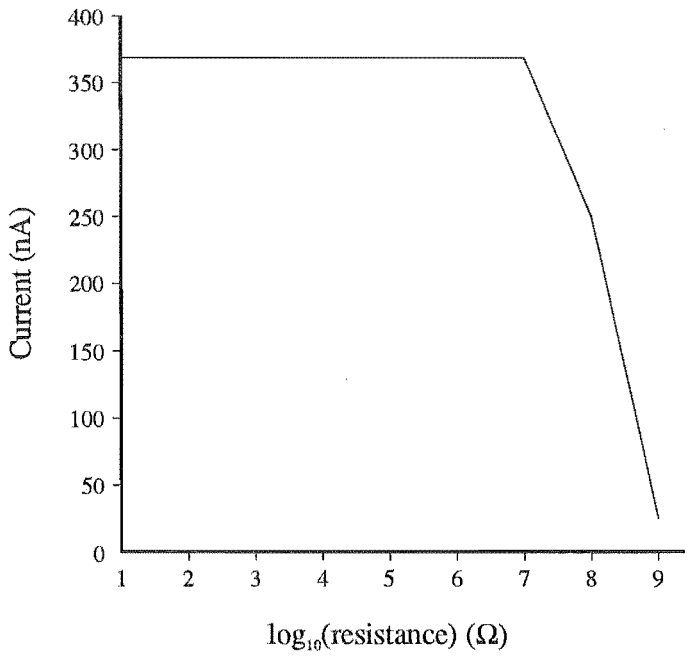
5.3.1 Very Low D.C. Current Source

As current is passed between electrodes, metal ions are dissociated into the solution. This can change the resistance between the electrodes which causes the current to change. A constant current source was designed to overcome this effect.

The circuit of Figure 5.2(a) shows a Widlar current mirror [Horowitz and Hill 1989]. This configuration was used for the constant current source apparatus. The Ebers-Moll equation was used to find the current through the transistors. The resistor R2 allows



(a)



(b)

Figure 5.2 (a) Widlar current mirror used to generate very low constant currents. (b) Widlar current mirror output versus resistance between the electrodes.

a much lower current in Q2 than Q1. This gives a very low steady current through the electrodes. From the Ebers-Moll equation it was found that the current would increase 4% when the current sources were taken from room temperature to the body temperature level of tissue culture incubators. This was taken into account when they were calibrated. Due to the required device accuracy, a matched pair of transistors

were used for Q1 and Q2.

To allow the current source to be set to any desired level a 220kΩ variable resistor was used on the controlling arm of the circuit. The circuit was powered by a 3V long life lithium battery. The current sources were constructed on veroboard and then sealed with temperature stable polyurethane to prevent the warm humid conditions inside the incubator affecting their performance.

The variation of current with changes in the resistance between the electrodes is shown in Figure 5.2(b). The circuit gave a good response for electrode resistances up to 10MΩ. Since the typical resistance between electrodes was approximately 10Ω, this allowed for many test dishes to be arranged in series. A number of current sources were constructed to cover a current range from 100nA up to 1mA.

At very low currents and electrode resistances the noise voltage is of the same magnitude to the current being measured as shown in Figure 5.3. The currents are near the theoretical limits of measurable signals. An ammeter was used that was able to accurately represent currents down to 100nA with a resolution of approximately 20nA.

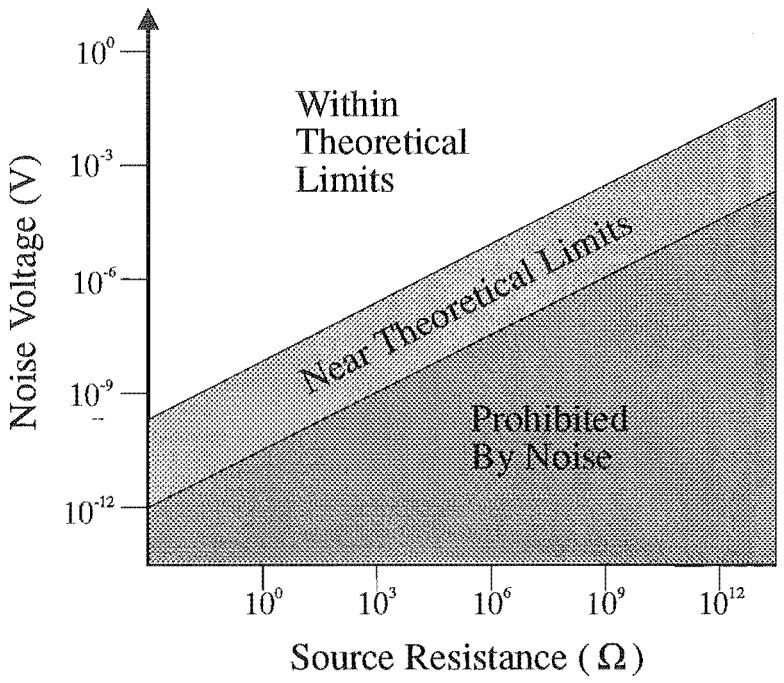


Figure 5.3 Noise voltages for measured signals and limits of practicality.

5.3.2 Electrode/Culture Dish Arrangement

All the experiments were performed in-vitro in sterile petri dishes. Two basic types of electrode design were built. One had parallel plates which maximized the area of

consistent electric field and the other had circular electrodes which maximized an area of known electric field change. These are shown in Figure 5.4. Both types of electrode design were attached to lids of standard 36mm petri dishes. This made the electrodes easy to clean and sterilize and easy to insert into cultures. Cultures were prepared in standard dishes (with their own lids). To insert the electrodes the standard lid was discarded and replaced with the electrode lid. For the parallel plate design a number

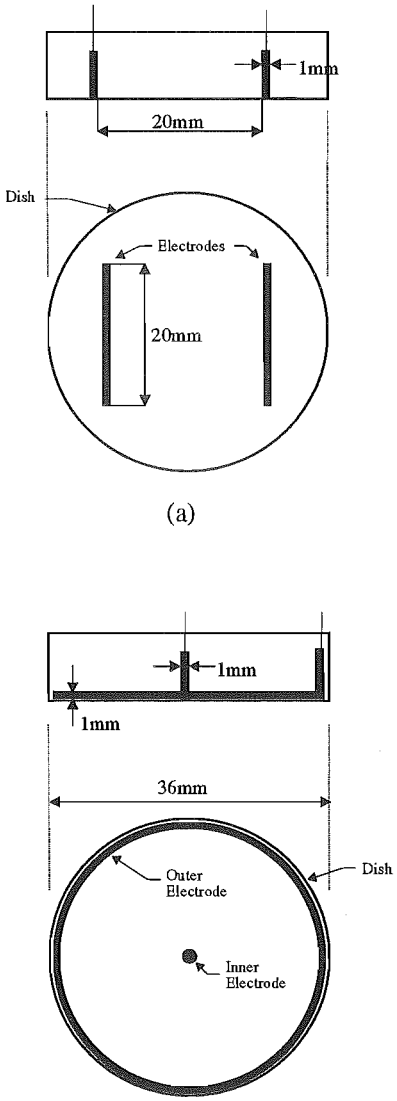


Figure 5.4 (a) Parallel plate electrode geometry. (b) Pin to ring electrode geometry.

of electrode pairs were constructed of silver, copper, aluminium, stainless steel and titanium. For the circular design the outer electrode always consisted of titanium, while the inner electrode consisted of either silver or titanium.

5.3.3 Cell Cultures

Three cell lines were prepared for study. These were of different origin and morphology which provided a means of comparison in reaction to stimulus.

U937, a human myeloid leukaemia cell line of monocytic origin was cultured in suspension in RPMI medium containing 10% fetal calf serum (FCS) and antibiotics. It grew continuously in culture and was subcultured 1:10 with fresh medium every 3-4 days.

COS-7, a primate kidney fibroblast cell line transformed by SV40 virus was cultured in Dubecco's Modified Eagles Medium (DMEM) and 10% FCS with antibiotics. It is an adherent cell line and was grown in sterile petri dishes, then subcultured by treating with 0.5% trypsin in medium (without FCS) and replating 10% of cells in fresh medium. Subculturing was performed once every 4-5 days.

MM96, a metastatic human melanoma cell line was cultured in Alpha Medium, 10% FCS. The melanoma was an adherent cell line. It was subcultured in the same manner as for COS-7 cells.

5.3.4 Experimental Procedure

All of the equipment (tweezers, trays, electrodes) was cleaned and sterilized using 100% ethanol under sterile conditions. Cell lines were cultured and/or transferred to the petri dishes and incubated until a large number of cells covered the dish bottom, but were not overcrowded. This was done over a 3-5 day period. Also under sterile conditions, the electrodes were placed in the prepared culture dishes and the current sources connected and turned on. The current sources were then set to the required level.

The cultures were incubated in two groups at 37^0C, 5% CO_2 for six and twenty-four hours respectively.

In one group, after six hours of incubation the electrodes were removed and the cultures observed and tested. The growth media was replaced with fresh media and the cultures were then replaced in the incubator. 18 hours later, and 24 hours after that, they were observed and tested again.

In the other group, after 24 hours of incubation the cultures were observed and tested with the electrodes still in position. The cultures were then returned to the incubator and 24 hours later, (and 24 hours after that), observed and tested again.

If at any stage the cultures became infected or were 100% non-viable then the test was concluded and the cultures discarded.

The Trypan Blue Dye Exclusion test is a method of obtaining an accurate account of cell viability in a sample. This process is destructive to the test sample. This meant that the test could only be used for non-adherent cell lines for which a small sample could be taken without destroying the culture.

For the experiments the test was performed as follows.

1. A 50μl sample of cell culture was mixed with an equal amount of 0.4% Trypan Blue.
2. A sample was then taken from the resulting mix and viewed by light microscopy on a haemocytometer.
3. Two cell counts, of 20 plus cells, were averaged and the result used to represent the total cell culture viability.
4. The sample was then discarded.

For the adherent cell lines, morphology was used to determine viability. Morphology studies the physical cell properties such as size, shape, cell structure, distinctness of cell components and general cell appearance. Though generally not as accurate as the Trypan Blue test, it provided results that were consistent with those for non-adherent lines.

As a comparison for study, control cultures were grown. The cultures were set in the same size dishes and of the same parent culture as the other test samples. They had no electrodes placed in them. These cultures represented normal cell growth for the culture and were used to ensure that any effect that was seen was due to the electrodes and/or current and was not normal cell activity for the culture.

5.4 RESULTS AND DISCUSSION

5.4.1 Cell Viability

The first experiment was designed to test the toxicity of the various metal electrodes upon cells. Leukaemia cells were used which grow suspended in the growth media. A range of currents from 0 - 0.5 mA were passed through the test dishes while they were being incubated. At periodic intervals, a small sample (50μl) of cell suspension was removed and mixed with an equal volume of 0.4% Trypan Blue. Dead cells were stained blue while viable cells remained clear. Cell counts were then performed on a haemocytometer.

After 80 experiments that covered all the metals over a wide current range for a 4 day period, some general observations were noted.

Aluminium had little effect on cell viability at any current level. However, when the ions were introduced to the highly ionic growth media, a cloudy precipitate was formed (probably $AlCl_3$). This precipitate obscured observation through a microscope. Due to this, aluminium was not used again.

Stainless steel was also reasonably inert. The death rate was approximately 3-4% higher than that of the control which had no electrodes in it.

Copper killed all cells in a very short period of time (less than six hours). This occurred even when no current was passed between the two electrodes.

Titanium had no effect on the viability of the cells. This was true even at the highest (1mA) currents. Research [Becker 1970, Duncan and Mattingly 1975] has shown that titanium has no ion diffusion for electrode potentials from 0-2V with respect to the electrode surroundings. Due to this experimental result it was decided to use titanium as a reference electrode in further experiments.

Silver's effects on cell viability are shown in Figure 5.5. A high number of cells were alive at 0nA due to the small amount of ions being diffused. Death over time occurred due to the formation of silver salts, which are toxic (in-vivo, these salts would not be formed and death is unlikely to occur). The viability drops at 100 and 200nA, then increases in the 300 - 400nA range. This is postulated to be the current range that affects cell growth, that is, dedifferentiation [Becker 1990, Liboff and Rinaldi 1974, Smith and Best 1989]. The increased viability could be due to the less mature cells being hardier and more resistant to death than the more specialized or mature cells [Albert 1988]. Some of the live cells had a much more distinct nucleus than the original culture cells which indicated a change in cell function. This is investigated further in Section 5.4.5. From 500nA upwards, cell viability dropped significantly.

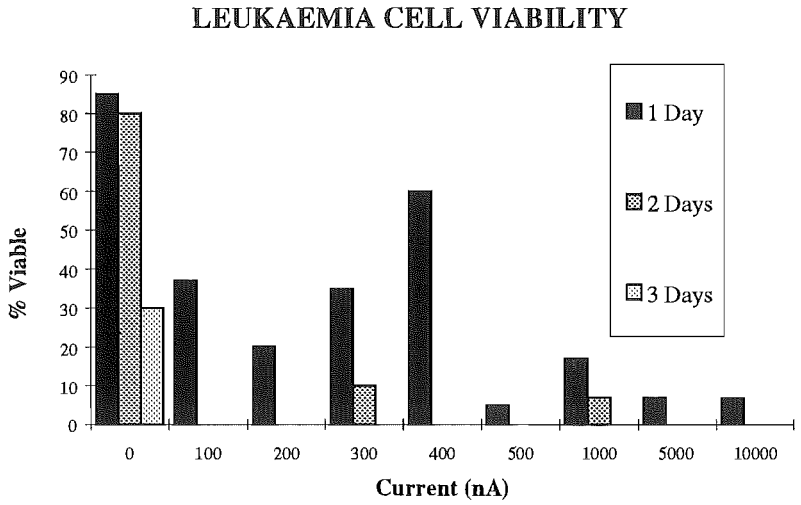


Figure 5.5 U937 cell viability as a function of exposure time and current levels.

These same experiments were performed on the melanoma and the primate fibroblast cell lines. Very similar results were found with increased cell viability in the 300 - 400nA range when silver electrodes were used.

From all the experiments, two metals were selected for further study; silver for its effects at 300 - 400nA, and titanium to act as a control as it had no toxic effect on the cells which would obscure the results.

5.4.2 Silver Disinfection

From the cell viability experiments it was apparent that the silver system had substantial anti bacterial, yeast and fungal properties. The other metals, apart from copper, showed very little or no disinfection characteristics. However, it was found that copper only had major toxic effects against the eukaryotic cells under test which was considered undesirable in the dedifferentiation experiments.

The disinfection and especially antibacterial effects of silver have been known for many years [Sykes 1958, Spadaro 1988]. Before it became economically more viable to use chlorine, silver ion disinfection systems were not uncommon. Silver nitrate is still sometimes applied to the eyes of newborn infants to avoid gonhorreal infection. The mechanism of silver ion disinfection is believed to be associated with the ability of silver ions to bind to biologically important molecules thus altering them to an extent that they can no longer perform their biological function. Further, the affected molecules do not always cause a bactericidal or fungicidal process, rather the cells lose their ability to grow and divide.

Due to its popularity, chlorine disinfection is becoming less effective as strains of chlorine resistant organisms are becoming more common. Other disinfectants, such as phenols, can be used but are often toxic to higher animals [Ranga Rao 1978]. Therefore, although more expensive, silver ion disinfection systems are beginning to be used again [McLean *et al.* 1993, Hamilton Miller *et al.* 1993]

Only very low numbers or concentations of silver ions are required for effective disinfection. This means that less physical material is needed which is an extra benefit when the weight and volume of the disinfection material needs to be as low as possible. For this reason, a silver ion drinking water disinfection unit is used in the NASA space shuttles.

Silver ion disinfection of drinking water has another advantage over chlorine treatment. Silver is odourless and tasteless. Therefore, the aesthetics of fresh drinking water are not lost after disinfection. The low volume and weight and palatable properties make silver ion disinfection of drinking water an attractive process for mobile applications, such as required by trampers or for where there is inadequate treatment of local drinking water which is prevalent in third world countries.

Initial experiments were performed on Y10/90 E-coli bacteria (amplicillin resistant) as an initial investigation into developing a portable silver ion based drinking water disinfection unit.

5.4.3 Silver Disinfection Experiments

Domestic tap water with a resistivity of 100Ωm and sterile distiled water with a resistivity of $10^4\Omega$m were innoculated with Y10/90 E-coli bacteria to a concentration of 10^3-10^4 cells/ml. The water samples were passed through a plastic chamber containing

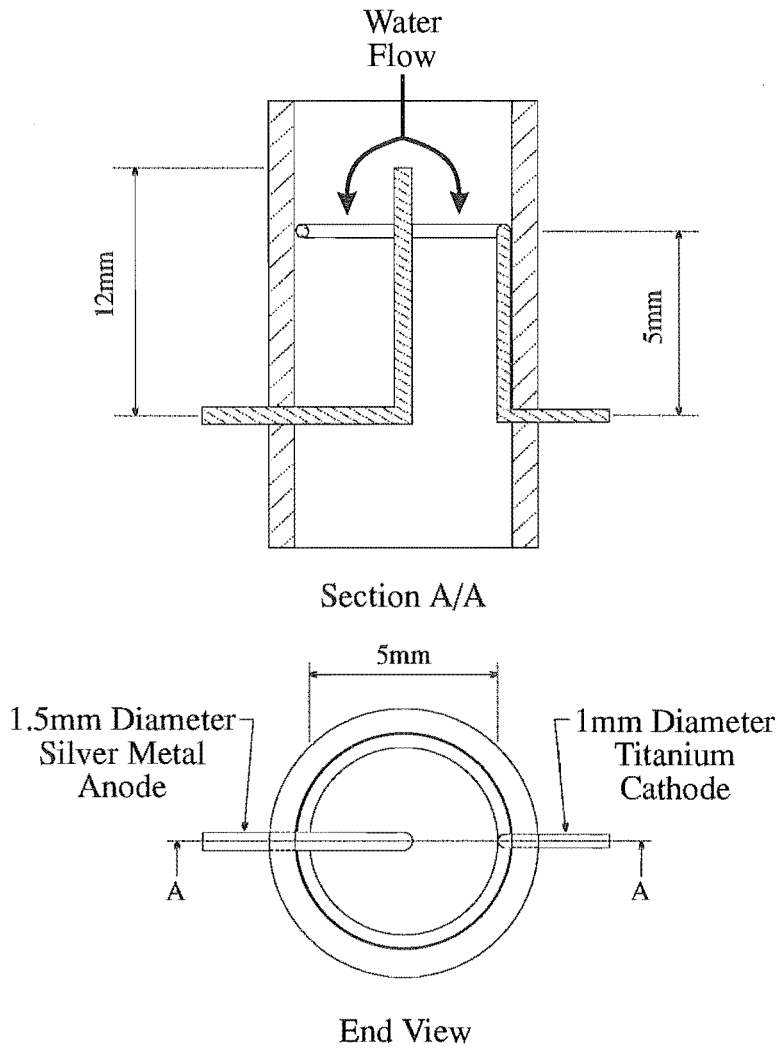


Figure 5.6 Silver disinfection electrode/chamber arrangement.

silver and titanium electrodes, as shown in Figure 5.6, at a flow rate of 7ml/min. A constant current of 8.7μA was applied to the electrodes so that the silver electrode gave off silver ions. The combination of flow rate, current and silver electrode surface area determined the silver ion concentration in the water. Furnace atomic absorption spectroscopy showed this to be at about 40μg/l which is below allowable first class drinking water levels [Pontius 1990]. After a determined standing time, a 1ml sample was centrifuged at 13000rpm for 2min. The supernatant was removed and the cells were resuspended in 0.5ml of sterile double distilled water. They were then plated on an amplicillin antibiotic agar plate and incubated overnight at 37^{0}C. The colonies were counted and referenced against a control sample that was not treated with silver ions. Colony counts versus standing time of a typical test is shown in Figure 5.7.

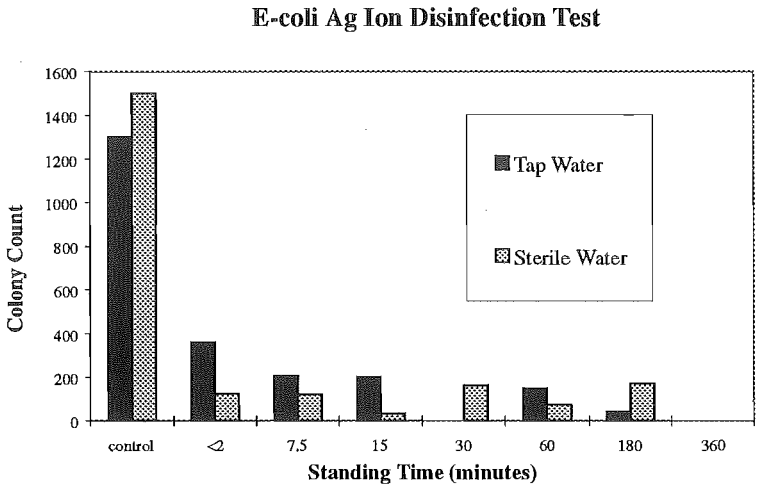


Figure 5.7 Resulting colony counts of E-coli bacteria versus standing time before washing in double distilled water.

The ion concentration is directly dependent on the flow rate. Therefore, if slower or faster flow rates are used, the current levels and/or electrode geometry must be varied accordingly. Standing times for effective disinfection levels will vary with each particular biological contaminant and its concentration and the ionic content of the water supply. Any system developed must then cater for a worst case situation where a large contamination of the hardiest organism that might be encountered, in a highly ionic water source, has to be treated.

Reports on the pathological diversity of silver ion treatment and the experiments performed, indicate that effective and efficient portable silver ion water disinfection units could be developed if there is little time constraint on standing time.

5.4.4 Dielectrophoresis

In the cell viability experiments of Section 5.4.1 clumping of the cells was noted. This was hypothesised to be due to dielectrophoresis [Pohl 1978] although this is not normally recorded to occur at such low electric field levels. In these experiments the dielectrophoresis occurred at field strengths of $1\mu V/cm$.

Dielectrophoresis was introduced in Chapter 3. It was shown that charge carriers in cell membranes experience forces in the presence of an electric field [Pethig 1988]. This causes a buildup of carriers on each side of a cell. If the field is non-uniform this usually produces a large concentration of charge carriers on one side of the cell relative to the other side. This results in a net force acting upon the cell causing it to move. This force must be large enough to overcome any random thermal motion or electrostatic forces.

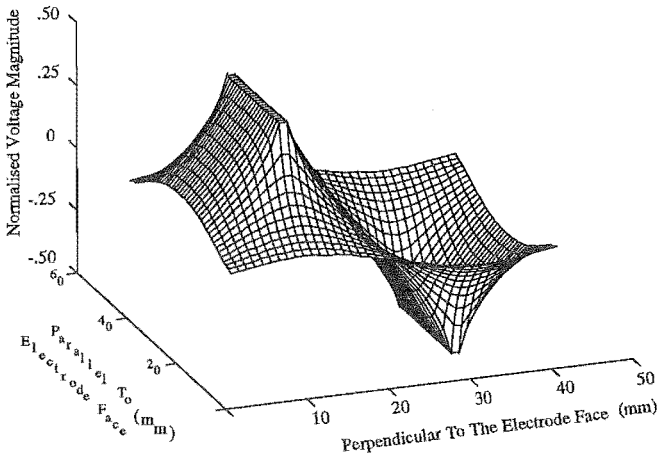
To test if this did explain the observation in Section 5.4.1, titanium electrodes were used to pass various currents through test dishes. Due to the resistance of the growth media, a voltage is developed between the two electrodes. Figure 5.8(a) shows the magnitude of this voltage, with the positive electrode on the left. By taking the derivative of the voltage function, the electric field can be found. This is shown in Figure 5.8(b). It can be seen that the areas of highest field magnitude are around the ends of the electrodes, then along the front of each electrode and then between the two electrodes. Over time, it was found that the cells would clump initially into the area corresponding to the highest electric field magnitude. As this area became full, new cells would clump in the area of the next highest field magnitude. Clumping around the ends and fronts of the electrodes was apparent in under six hours.

Cells have a net charge on their membrane due to an ion exchange with their surroundings that regulates their growth. If the cells were moving due to their charge (electrophoresis), there would have been a buildup of cells at one electrode only. The clumping of cells around both electrodes showed that dielectrophoresis was occurring.

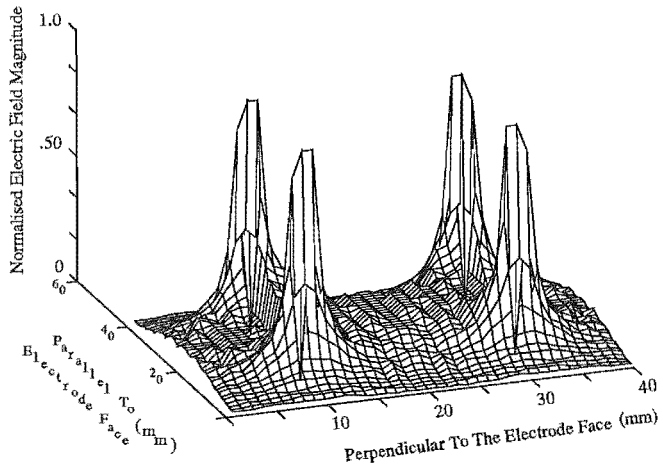
To further investigate dielectrophoresis, some new electrodes were constructed. These had a circular outer conductor, and a point as the inner conductor as shown in Figure 5.4(b). The use of small radius inner conductor gave a high field strength in the centre of the dish. Cells were observed moving toward the centre electrode irrespective of whether it was positive or negative, or whether a 500Hz a.c. signal was used, thus confirming the occurence of dielectrophoresis.

5.4.5 The Effect Of Silver Ions On Cellular Growth

This experiment was designed to test the effect of silver ions on the cellular function of the melanoma, myeloid and primate fibroblast cells for comparison. To combat the effects of the toxic silver salts that are normally formed, the test dishes were treated for only six hours at 300 - 400nA. The electrodes were then removed and the growth



(a)



(b)

Figure 5.8 (a) Normalised voltage magnitude between the parallel plate electrodes. (b) Normalised electric field magnitude between the parallel plate electrodes.

media was refreshed. Melanoma adheres to the dish so is left behind when the growth media is removed. This meant that the silver ions that had penetrated through a cell membrane were left behind and could still interact with the cell. A visual observation of the cell morphology (size, shape and other physical features) was then carried out.

Some melanoma cells became non-adherent. These cells had a much more distinct nucleus than the initial culture cells and were rounder in shape as shown in Figure 5.9. This was analogous to the effect observed with the leukaemia line. The primate fibroblast yielded a very similar result. These results are significant in that all three,

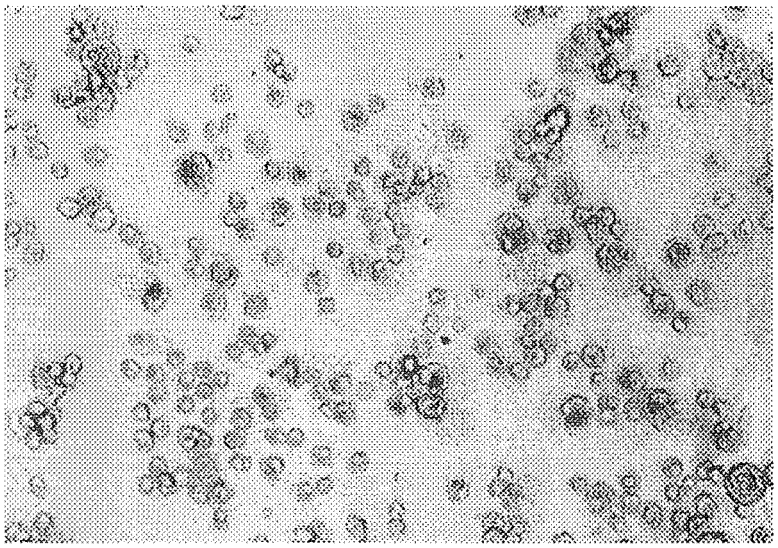


Figure 5.9 Uncharacteristic non-adherent melanoma cells.

initially very differently shaped cell types took on the same rounded and well defined nucleus morphology when treated by silver ions. This may indicate that they have returned to their respective common predecessors. That is, they have dedifferentiated. These predecessors may be one of the multipotent or even rudimentary pluripotent cells outlined in Figure 5.1. The specific stage of cell maturity could not be determined. The affected areas were only around the electrodes. The positive electrode had a much greater area of effect than the negative electrode.

If dedifferentiation has occurred, then depending on the degree of regression, it should be possible, with the appropriate biological controls, to turn these cells into whatever cell was desired. When the silver electrodes were removed, within two days, the changed cells in the region the negative electrode had occupied, grew back to their initial morphology. Their biological control could be other specific cells already in the solution. The mechanism of shared cell information for this would suggest that the cells are operating as a metazoan culture [Goodenough 1984]. This is uncharacteristic of cancers which normally operate as a protozoan culture. That is they operate independently of one another. This is another possible indicator that dedifferentiation had

occurred.

Cells grew back much more slowly in the area which the positive electrode had occupied. This indicates a much greater concentration of silver ions in that area. The lack of growth may indicate that the cells in the area were being exposed to higher levels of toxic solutions generated by the silver ions.

Some affected cells became non-adherent and clumped together in groups as shown in Figure 5.10. These cells were originally thought to be dying. However, the cells may be grouping together in a form of pseudo-tissue [Becker 1985a]. If the cells were dead, the membrane would become more permeable. This would allow the growth media to enter the cell. The cell would then have the same internal ion concentrations as the external growth media, and they would subsequently have no attractive forces between them. This is substantiated by the observation that the few non-viable cells in the medium appeared stationary. It is therefore thought that the attraction is more biological than electrical.

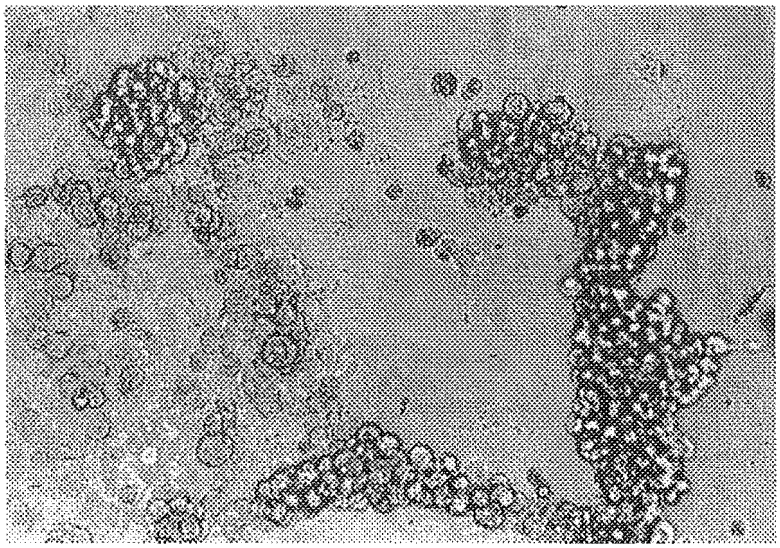


Figure 5.10 Clumped melanoma cells possibly forming a pseudo-tissue.

A further investigation involved the use of circular electrodes as shown in Figure 5.4(b). These had a silver centre conductor and a titanium outer conductor. Dielectrophoresis continually moved the cultured leukaemia cells toward the centre. Silver ions were continuously flowing from the centre to the outside. As the cells encountered the silver ions, they changed as detailed previously. Following this change, continued exposure to the silver ions resulted in cell death. The cells then moved away from the high field strength area, collecting in the area of lowest field strength. This change in direction may indicate a change in cell dielectric properties, leading to negative dielectrophoresis. It may also indicate a taking up of positive silver ions, causing an

electrostatic attraction between the cell and the outer negative electrode.

5.5 CONCLUSIONS

The effects of passing low d.c. currents through various metallic electrode pairs suspended in solution on biological cell viability and morphology have been investigated.

It has been found that silver ions have unique effects on cell viability relative to the other tested metals. These effects were exhibited as disinfection and possible dedifferentiation properties.

The disinfection characteristics were investigated and it was found that it may be viable to develop a portable silver ion based drinking water disinfection unit. The major drawback is determined by the long standing period required during operation.

A noticeable effect was observed when the mammalian cells under examination were placed between electrodes in conventional growth medium and a current of between 300 - 400nA was applied. This effect may be dedifferentiation as indicated by the change in morphology in the cell types examined. Under treatment with silver ions and d.c. current, the three very different cell morphologies changed to a common one. This suggests that they are returning to a more primitive or immature state. When the electrodes were removed from the solutions, the effect was quickly reversed. If dedifferentiation was the observed effect then the cells re-differentiated back to their original state.

A consequential observation was that the phenomenon of dielectrophoretic motion of cells was seen to occur at very low electric field magnitudes. Dielectrophoretic motion is usually required to work over a time frame of seconds. In these experiments, observations were recorded over a period of twenty-four hours.

If cells can be dedifferentiated in-vivo, then it would be possible for the body to influence them to become the appropriate cell type for their location. This has important consequences in that a cell which has become cancerous by the expression of a surplus gene could be dedifferentiated. The body could then turn it back into a normal cell through normal differential regulation which would repress the previously active oncogene. This could be a non-surgical technique for treatment in the initial stages of skin cancers such as melanoma. This is the next step in this research.

Chapter 6

RESONANT EM ENERGY TRANSFER INTO BIO-SYSTEMS

6.1 INTRODUCTION

Electromagnetic (em) resonant energy transfer into biological systems is used extensively in medicine. However, most applications are based on diagnostic processes. By far the greatest number of applications are in medical imaging and spectroscopic analysis [Morris 1986, Lange *et al.* 1989, Hollas 1982, Swartz *et al.* 1972]. Much less research on possible therapeutic applications of em resonant energy transfer has been carried out.

Electromagnetic fields are used in some reasonably effective therapeutic applications. For example, treatment of cancer by microwave, X-ray or γ-ray radiation is in common use [Field and Franconi 1987, Swan 1981, Mansfield 1983]. The mechanisms of action for these therapies do not have any resonant attributes. Therefore, very little or no physical specificity exists in the treatment. Most of the beneficial effects are determined by the biological functions available to the treated organisms. Herein lies one of the major problems experienced in many medical treatment regimes. Poor specificity towards the pathological component results in low efficacy and often marked undesirable side effects. Resonant energy transfer mechanisms may be able to overcome the targeting problem in many instances.

Resonant characteristics are determined by extremely specific physical parameters and any deviation from these parameters, changes the resonant condition. Thus, if certain em resonant effects are observed in specific biological components of interest, then they may facilitate a method of targeting based on physical conditions. Certain therapies could then either use the em energy transfer directly or use the resonance condition as a marker for another therapeutic agent.

This chapter looks at what constitutes em resonant energy transfer, how this affects biological systems and what therapies may be devised as a result.

6.2 RESONANT EM ENERGY TRANSFER

Energy transfer from an em field into a material involves the conversion of em energy into excitation energy in the material, usually exhibited as mechanical energy. The actual mechanisms of energy transfer are dependent on the level of energy and material involved. The unifying factor is that mechanical forces are generated by the interaction of an em field with electric charges in the material.

All physically restricting bonds, whether they are directly mechanical such as those found in a construction like a bridge, or electrostatic such as those found in molecules, can be modelled with combinations of appropriate second order differential equations. Thus, it would be expected that all physical bonds have characteristic mechanical resonances. These resonances may or may not be observable depending on the amount of effective damping. Given a set of environmental conditions, the resonances will occur at particular frequencies of oscillation. Excitation with energy at these frequencies results in resonant energy transfer.

Forces generated in materials by em fields will exhibit resonant energy transfer at particular frequencies. The sharpness, magnitude and stability of the resonance transfer is dependent on the mechanisms of transfer and condition of the local environment. Generally, as the overall size and complexity of a particular structure increases, the resonance frequencies tend to decrease [Rao 1990]. For example, a building measured in tens of meters may have an overall mechanical resonance at 1Hz, whereas a crystal lattice measured in nanometres may have an overall mechanical resonance at 100MHz. If an em field is used for resonant energy transfer, then as the frequency increases, so does the relative energy involved, due to the relation,

$$E = hf \tag{6.1}$$

where E is the energy of an em field, h is Planks constant and f is the em field frequency.

The forces generated may try to induce vibration or rotation in the structure or material. These modes of energy transfer can be described by characteristic equations. Full analysis of these equations can be found in many physics and spectroscopy texts [Kittel 1986, Sears *et al.* 1982]. For em field energies above about 0.1eV, resonant absorption may be experienced when exciting an electron to a higher energy level. When the energy of applied em fields reach about 5eV, then direct atomic resonance energy transfer begins to occur. This is initially exhibited as the ejection of valence electrons resulting in ionisation (photoelectric effect). At even higher energies, other effects such as scattering (Compton effect) and pair production may also occur [Sears *et al.* 1982, Friedlander *et al.* 1981].

It is important to note that over all these energy ranges, no effect has strict upper or lower bounds and may overlap other effects.

6.3 RESONANT EM ENERGY TRANSFER INTO BIO-SYSTEMS

In a practical sense, biological systems and their components range in scale from around a few tens of metres down to atomic levels. Thus it would be expected that physical resonances may range from sub hertz to γ-ray frequencies. It would also be expected that many of these resonances could be excited by the application of appropriate em fields. A number of investigations [Frohlich 1988a, Liboff and Rinaldi 1974, W.H.O. 1984] have shown that this is indeed the case.

As previously mentioned, most em resonance energy transfer applications are based in diagnostic processes. However, research has shown that significant biological effects can be produced due to em resonant energy transfer [Frohlich 1988a, Liboff and Rinaldi 1974]. The energy (or frequency) and power of the excitation em fields determine what effects occur. It may be possible to develop certain effects into components of medical treatments.

6.3.1 High Energy Effects

In the context of this section, 'high energy' is defined as ionising levels and above, or greater than about 5eV.

Electromagnetic radiation above 5eV can be resonantly absorbed by numerous mechanisms. The resonant absorption may result in exciting an electron state, ejecting an electron or raising the energy level of an atomic nucleus.

High energy em radiation is utilised in some cancer treatments and is usually labelled as radiotherapy. This is in itself an exceptionally complex field of study and a great deal of theory is associated with it [Swan 1981, Hoppe *et al.* 1983, Mansfield 1983]. However, the common mechanism of effect is based on the higher efficiency self repair attributes of normal cells compared to their cancerous counterparts. Thus, for a given radiation dosage which results in some form of cellular damage, normal cells are more likely to survive the exposure. For any improvement in these types of radiation therapies, resonant energy absorption characteristics must be incorporated. The task therefore, is to find resonant energy absorption peaks that are specific to atoms preferably found only in the cancer cells.

If X-rays or γ-rays are resonantly absorbed by atoms specific to the cancer cells, then when the electrons are recaptured or the nucleus relaxes, those same atoms become radiation emitters. Cells in close proximity to these atoms (especially the radiating atom carriers) receive an extra radiation dose. This should result in a larger survivability divergence between normal and cancer cells. As the main atomic differences between normal and cancer cells are in their molecular makeup, then any resonant effects must be sensitive to this difference. Molecular spectroscopic techniques utilising high em energies might provide a means to accomplish the required sensitivity and

specificity.

Two major spectroscopic processes known as extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge structure (XANES), show variation in X-ray absorption in the vicinity of an X-ray absorption edge due to the atoms surrounding the absorbing site [Koningsberger and Prins 1988]. Thus, if the molecules pertaining to the cancer cells can be isolated and tested using EXAFS or XANES, then it could be possible, by using the obtained absorption information, to produce more efficient energy transfer into those molecules and hence cells. This should result in a greater pathological diversity between exposed normal and cancer cells.

A spectroscopic technique exists which utilises γ-radiation. It is known as Mossbauer spectroscopy. This process is extremely sensitive to γ-ray energy and the molecular environment of the absorbing atoms nucleus [Gonser 1981]. Unfortunately, Mossbauer spectroscopy is currently limited to looking at the characteristics of ^{57}Fe in biological applications. However, if γ-ray lasers are brought to full development, it may be possible to investigate any atoms molecular environment. This could again give information about what γ-ray energies would result in more efficient absorption for molecules specific to cancer cells.

Due to the extensive side effects that can result from radiotherapy, only extremely pathological conditions can be considered for treatment in that manner. In addition to cancer therapy, other prime candidates are AIDS therapy and chemical detoxification.

The HIV virus produces highly recognisable protein molecules that do not often change due to viral mutations [Greene 1993]. Thus, by the arguments above, it should be possible to obtain effective treatment of this disease by selective high em energy transfer into those molecules. The cell kill rate diversity between the virus or virus infected cells and normal cells would have to be relatively substantial as whole body irradiation would be necessary owing to the spreading nature of the disease. This is highly probable for the virus, as the viral RNA reverse transcriptase will not be able to repair all of the RNA breaks induced by the radiation which should result in a much lower virus viability. The haematopoietic cells targeted by the HIV virus are known to be by far the most sensitive to high energy em radiation and can be completely irradicated by X-rays without severe damage to the rest of the biosystem [Roitt *et al.* 1985]. If an appreciable difference in sensitivity of the HIV virus or infected blood cells to normal blood cells to a chosen radiation energy exists, then an effective dosage may be of the level that significantly fewer side effects might be experienced.

Contamination of a person by a slow acting but lethally toxic chemical could in some instances be treated by the described processes. This would be especially true if the site of molecular activity involved an atomic element that does not normally occur in biological systems.

X-rays and γ-rays can traverse entire bodies, enabling simple application of the radiation to the required areas. Lower energy em radiation such as far UV which still

may be useful, might not be able to effectively penetrate inner body regions. In these situations, some sort of emitter must be placed appropriately near to the treatment area. This may require invasive implantation.

6.3.2 Low Energy, High Power Effects

For em energy below ionising levels, at reasonably high power densities (measured in W/cm^2), the main mechanism of energy transfer is thermal in nature and has non-resonant characteristics [W.H.O. 1981]. Energy transfer of this type is used in medicine for hyperthermia cancer therapy and diathermy [Field and Franconi 1987].

Hyperthermia treatment of cancer usually utilises beams of microwave radiation focused on tumour sites within the body. This locally heats the tissue to relatively lethal levels (around 40-45^{0}C). More efficient hyperthermia kill rates are achieved when the tumours are also exposed to X-rays.

Hyperthermia has major inherent problems that reduce its efficacy. If treated tumour cells survive, then they become thermally insensitive relative to normal tissue for a time span of days. This problem can not be simply resolved by generating excessive heat so that no cells could possibly survive, as this would result in massive killing of surrounding normal tissue. There is a thermal window that is determined which states the maximum allowable amount of normal tissue that can be sacrificed. In some instances, the window is too small for the hyperthermia technique to be of any use. Associated to this problem is the limited achievable resolution of the microwave beams.

The wavelength of the microwave radiation limits the beam resolution to around $1cm^2$. On cellular or even tumour scales, this is large which makes it difficult to target the tumour and its boundaries accurately. The result is an unavoidable direct exposure to a significant number of normal cells.

The microwave radiation used, acts by the dielectric heating of water in body tissue. Therefore, the only variation in heating levels is determined by the water content of the exposed tissue. Unfortunately, most tumour masses have practically the same water content as surrounding normal tissue.

If energy could be resonantly coupled into the tumour cells, then this could lessen the problems associated with the thermal window and targeting. Once again, the main physical difference between cancer and normal cells is in their molecular makeup. There are numerous ways to induce resonant coupling of non-ionising energy into molecules which may involve NMR [Hollas 1982], ESR [Swartz *et al.* 1972] or IR [Hollas 1982]. However, there is an effect of power spreading of resonant absorption peaks that severely limits the power that can be usefully coupled into specifically targeted molecules. Also, from microwave levels up to UV, the depth of em penetration reduces with increasing frequency. This is an important factor in determining the positioning of em energy sources.

6.3.3 Low Energy, Low Power Effects

Low energy is again interpreted as lower than ionising levels. Low power in this section is any power density that does not result in appreciable thermal effects relative to biological systems. That is, less than about 0.1^0C variation in temperature.

Investigations have shown that cells emit em radiation at kHz-MHz [Pollock and Pohl 1988], millimetre wave [Kremer *et al.* 1988], IR and visible [Inaba 1990] frequencies. These emissions are often coherent amongst cellular systems and exhibit resonant characteristics.

The kHz-MHz emissions are attributed to normal cellular metabolism and is probably a by-product of that activity [Pollock and Pohl 1988], although this is not definite. Millimetre wave emissions are quite different.

The actual mechanisms of millimetre wave generation within cells are mostly unknown. However, due to the apparent resonant and coherent characteristics and theoretical cellular transmembrane mechanical resonant frequencies, it has been postulated that the cell membrane is the site of generation [Golant 1989]. The inherent transmembrane potential can be considered as a dipole. If the dipole is vibrated longitudinally, then this will result in the generation of an oscillating em wave with the same frequency as the vibration. Viscoelastic mechanical analysis of the lipid bi-layer membrane has indicated that it probably has a transmembrane mechanical resonance in the region of 10^{11}Hz (millimetre wave region). If it is accepted that the millimetre waves originate from the cell membrane, then the questions of how the membrane is mechanically excited and why, are raised.

No known metabolic activity directly accounts for the resonant mechanical excitation of the membrane. It is likely that some specific mechanisms have been evolved to carry out this process. If this is the case, then there must also be a reason for it. Recent studies have indicated that cells may use high frequency em signals (millimetre to visible frequencies) for cell to cell communication [Frohlich 1988a, Golant 1989, Inaba 1990]. Communication of this form may be associated with immunological processes or growth and differentiation control. It has been suggested that cancer cell growth is accompanied and reinforced by changes in the high frequency em oscillations, indicating that a minimum number of abnormal cells are required for runaway growth [Frohlich 1988b]. Correspondingly, foreign invading bodies that induce an immune response, remotely alert immune system cells by their abnormal em emissions probably generated by their associated antigens.

Other reports have shown that low power millimetre wave radiation can be resonantly absorbed by DNA and affect its tertiary structure [Kremer *et al.* 1988, Belyaev *et al.* 1993]. Low power microwave radiation has been shown to have resonant effects on cellular growth and animal physiology and behaviour [Grundler *et al.* 1988]. Thus, it may be possible to beneficially affect the progress of disease by the application of

appropriate low energy and low power em fields. This would require understanding the em code or language cells use in their fight against disease. Whether this is an achievable goal depends on the validity of postulated ideas and the ability of researchers to emulate the complex yet extremely successful processes of Nature.

As an initial experiment in this area, a normally inert and biocompatible metal such as titanium or platinum could be placed within an organism and have a millimetre wave signal applied to it to see if an immune response is initiated against it. The frequency and power of the signal could be varied to see if there were any resonant immune response characteristics.

Looking more from a physical view point, em energy from microwave to visible frequencies could, theoretically, resonantly transfer into bio-molecules. The result may be rotation or vibration of molecule sections and bonds. These effects could alter the normal functioning of some molecules which may or may not be desirable. Experimental determination of possible effects on molecule function could be carried out using biologically isolated physical science methods which would greatly simplify interpretation of observed biological effects.

Resonance effects of em fields have been observed down to extremely low frequencies (about 50Hz) [McLauchlan 1992, W.H.O. 1984]. However, as the energy of the em fields are so low at these frequencies, it is likely that the observable effects are powered by secondary biological processes. Although demonstrable, effects of extremely low frequency em fields are extremely variable and difficult to reproduce. Also, the implications of the effects observed can not be easily ascertained and will probably require long individual experimental time frames.

6.4 DISCUSSION

If resonant effects are observed at different energy levels, then it may be possible to combine these to form some kind of treatment. In this situation, independently sub-useful effects could be combined to produce a successful therapeutic treatment. Alternatively, an already successful treatment may be improved by combining other resonant effects. A hypothetical example might be that a resonance effect is observed in atoms of interest with XANES and application of X-rays with the appropriate energy shows a statistically favourable result. In addition, ESR investigation has shown that particular atoms in the molecules of interest can resonantly absorb energy in the presence of a defined magnetic field when ionised. The combination of these effects may result in a larger ionised population of the targeted atoms, leading to an improvement in results.

It appears that Nature has chosen to use low energy and low power em fields in many of its functions. Mainly for this reason, it is the author's opinion that utilisation of these particular conditions will result in the most effective therapies and have the least unintentional impact on the biosystem. Nevertheless, research leading to effective

treatments is likely to take a substantial amount of time, effort and money. Therapies based on physical responses would probably require less of all aspects stated in the preceding sentence. Therefore, research in both areas is warranted.

Whatever the motivational preferences are for possible research, it should be expected that initial experimentation would be designed to validate simple assumptions. Extreme care will be required to avoid interpreting artefacts as biological effects, which can be a major stumbling block in biological investigations. This is especially true for lower energy em fields.

6.5 CONCLUSIONS

Modern therapeutic use of em energy is relatively limited and generally ineffective except in a few isolated situations. Very little consideration has been made to the possibility of using em resonant energy transfer.

Resonance characteristics facilitate the ability to specifically target biological components associated with conditions of interest. Therapies based on significant and reproducible resonant energy transfer are more likely to be effective and cause fewer side effects. Resonant energy transfer effects may also be combined or aid effective conventional therapies.

The mechanisms of resonant action are dependent on the energy (frequency) of the applied em fields. High energy effects are almost solely due to direct physical alteration of atoms which occur independently of the biological macro-environment. Lower energy effects are more subtle and influenced by biological environments and functions.

Natural em emissions from cells suggest that understanding and emulating those emissions may yield the most comprehensive information on the role of em fields in biosystems. Also, as a result, the true potential of resonant em energy transfer in therapeutic medical applications is likely to be found at those energy levels.

Chapter 7

CONCLUSIONS

The work of this thesis has shown that em fields can be used in biological applications for more than diagnostic purposes. The effects investigated indicate that there is a world of possibilities for electromagnetics in biology and medical treatments, as yet untapped. A concentrated effort in trying to find these applications should result in significant new discoveries.

In Chapter 2 and Chapter 3, the processes of electroporation and electrofusion were studied. Physical and theoretical models and preliminary biological testing found that the prevalently used d.c. pulse systems are fundamentally sub-optimal. Aspects such as asymmetrical breakdown, cellular rotation, heterogeneous cell and organelle size, and impedance changes which all directly affect operational efficiencies, are not sufficiently addressed.

The use of a.c. waveforms symmetrical about a zero potential axis should significantly reduce the inherent problems associated with d.c. pulses. In addition, changes to the current protocols used in multiple pulse situations could be made to maintain permeability states without major loss of cell viability.

Obtaining high efficiency electroporation and electrofusion results is often considered as almost an art. This is mainly due to uncertainties involved with only slight variations in biological and physical conditions, changing optimum electrical parameters. Using impedance information in a feedback situation to the breakdown electric field should eliminate much of the uncertainty.

The implications of improving electroporation and especially electrofusion processes by even a small amount, are significant. Human monoclonal antibody research would be the main benefactor and is set to become a multi-billion dollar industry. Efforts in transgenic research will also be aided.

Chapter 4 looked at the possibility of using the lysing effect observed in electroporation and electrofusion studies, to disinfect biologically contaminated liquids. Tests showed that it was indeed possible to effectively disinfect low conductivity water inoculated with serratia marcescens. Further investigations indicated that physical parameters of the liquid being treated could place limits on the applied electric fields and

hence effectiveness. Conductivity of the liquid was found to be a particularly strong influencing factor. High conductivity produces problems with electrolysis of electrode material and heating. Electrolysis can be reduced by using higher frequency a.c. waveforms although with limits. Above about 100kHz, the effect of biological membrane capacitance starts reducing killing efficiency. If a.c. waveforms are to be used, dead band regions must be kept to a minimum so that exposure of cells to breakdown electric fields for a practical amount of time, is assured.

If very low conductivity liquids such as kerosene are to be treated, then either the electrode geometry must be arranged so that membrane breakdown electric fields can be induced in the cells, or the conductivity of the liquid increased so that membrane breakdown electric fields can be applied to the bulk liquid. It is the successful adaption of the h.m.e.f. disinfection system to those low conductivity liquids that will most likely provide significant advantages over presently available processes.

The unique effects of combined low magnitude d.c. electric fields and silver ions on cancer cells were detailed in Chapter 5. Within a particular range, three morphologically different cell types were seen to take on a degree of structural similarity. They became spherical, non-adherent and had a distinct nucleus. These are characteristics of many stem or multipotent cells. Other metal ions were tested with the electric fields but did not reproduce the silver ion results. If dedifferentiation was induced, this will have major ramifications in the possible development of regeneration and new cancer treatments.

It was also noted that the silver ion system offered a possible method for the chemical disinfection of water supplies. Results showed that very low concentrations of silver can provide effective disinfection against E-coli bacteria, if the treated water is left for a few hours. Advantages over other disinfection chemicals were evident. Very low concentrations of silver could be used which in turn requires less material. The aesthetic qualities of the water are not affected by the treatment. Due to its uncommon usage, fewer strains of biological contaminates are likely to be resistant to it.

In Chapter 6, the mechanisms of resonant energy transfer were suggested to provide a method of targeting in certain biological applications. The mechanisms of action are energy and power dependent. Higher or ionising energy levels are more likely to work on direct physical effects, whereas lower energy and power levels are more likely to resonantly affect biological functions. It is speculated that lower energy and power resonant transfer is utilised by Nature in cell to cell interactions such as in the immunological system. Electromagnetic treatments in this area are then probably best looked for by using similar frequencies, time intervals and power densities.

7.1 FUTURE WORK

For the thesis material in general, substantially more research and experimentation is required on actual biological systems. In each chapter (bar Chapter 6), initial biological experimentation was carried out but not to any final levels.

To realise the effects of using a.c. pulsed systems in electroporation and electrofusion, physical apparatus must first be built that can produce the appropriate waveforms. Even though some block diagrams suggested possible apparatus configurations, a considerable amount of effort will be needed to design and build that apparatus. Once the equipment is available, experimentation on the actual biological benefits in using a.c. pulses for both electroporation and electrofusion can be carried out.

More comprehensive physical and biological experimentation is required in the h.m.e.f. disinfection work. The characteristics of frequency and conductivity on electrolysis of different materials needs to be ascertained. The results will positively indicate practical upper limits on liquid conductivities. Special effort is warranted in developing viable h.m.e.f. disinfection systems for the treatment of normally very low conductivity liquids owing to the potential advantages over current systems.

Investigations into the effects of combined low magnitude d.c. electric fields and silver ions have been relatively superficial. Further, in-vitro studies on transferring treated cells to other cell populations to see if there is sufficient differentiation information to change the treated cell type would be interesting. In-vivo studies may provide more spectacular results, although these experiments would have to be carefully constructed to avoid missing possible effects. If results are promising, then there exists enormous potential of further research in regeneration effects and cancer treatment.

The work associated with Chapter 6 has only just begun. This being the case, appreciably more work on the theoretical em resonant energy transfer mechanisms in biological systems is necessary before any well structured experiments can be conducted. The time taken to study theoretical aspects more fully should reduce the chances of looking for an effect that is in reality an impossibility.

Appendix A

ROD SHAPED CELL MODEL OF ELECTROPERMEABILIZATION AREA

This model assumes that a rod shaped cell is made up of three sections. A middle section is defined as a thin walled cylinder. Two end sections that cap the middle section are hemispheres with the same radius and wall thickness as the cylindrical section.

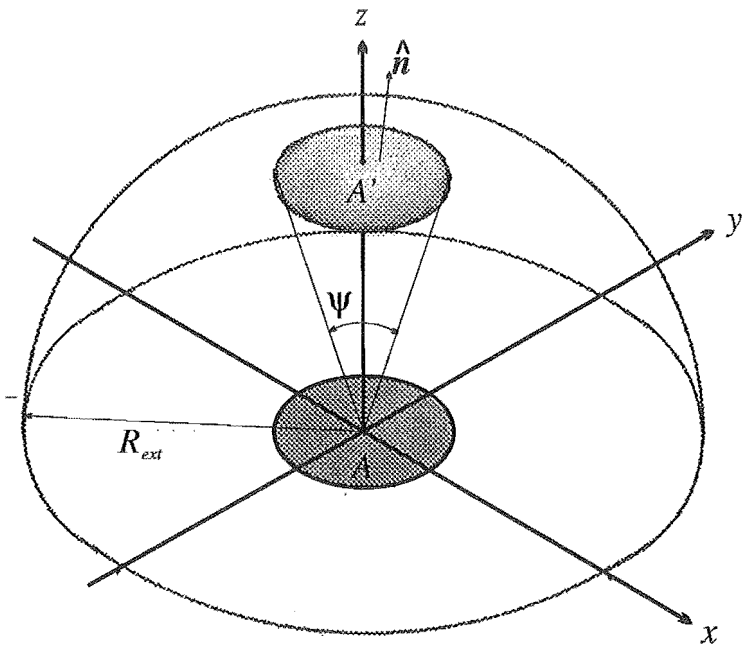


Figure A.1 Effective electropermeabilization area, A', defined for the hemispherical end cap sections

For any cell orientation in a uniform electric field, the two hemisphere sections will have the same electropermeabilization area A', shown as a polar cap region in Figure A.1 (only the outer surface is shown to reduce illustration complexity). An effective permeabilization angle, ψ, can be set for these polar caps. Using the notation shown

in Figure A.1, the equation of a sphere is,

$$z^2 + x^2 + y^2 - R_{ext}^2 = 0 \tag{A.1}$$

where x, y and z are the cartesian coordinate values and R_{ext} is the external hemispherical radius. The normal vector, $\boldsymbol{n}$, to this surface is,

$$\boldsymbol{n} = 2x\hat{\boldsymbol{i}} + 2y\hat{\boldsymbol{j}} + 2z\hat{\boldsymbol{k}} \tag{A.2}$$

where $\hat{\boldsymbol{i}}$, $\hat{\boldsymbol{j}}$ and $\hat{\boldsymbol{k}}$ are the cartesian coordinate unit vectors. Taking a unit element dA' and projecting it onto the x, y plane to form dA, as shown in Figure A.2, then,

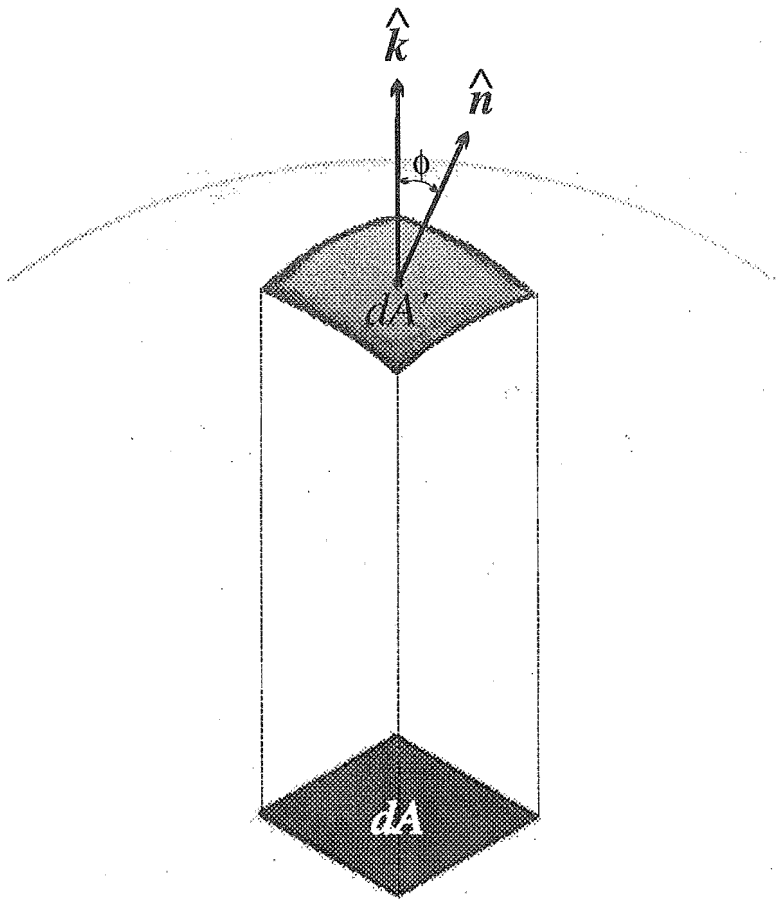


Figure A.2 Projection of the unit area, dA', onto the x, y plane

$$\hat{\boldsymbol{n}} \cdot \hat{\boldsymbol{k}} = \cos\phi \tag{A.3}$$

where ϕ is the angle between the unit normal $\hat{\boldsymbol{n}}$ and $\hat{\boldsymbol{k}}$. Also,

$$dA' = dA/\cos\phi \tag{A.4}$$

Thus,

$$A' = \iint_A \frac{dA \mid \boldsymbol{n} \mid}{\hat{\boldsymbol{n}} \cdot \hat{\boldsymbol{k}}} \tag{A.5}$$

Expanding Equation A.5 gives,

$$A' = \int_0^{2\pi} \int_0^{u} \frac{\sqrt{x^2 + y^2 + z^2}}{z} r\,dr\,d\theta \tag{A.6}$$

where $d\theta$ is the polar integral operator of angle, dr is the polar integral operator of radius, r is the variable of radius and u is the radius of the projected surface A. Substituting $x = r\cos\theta$ and $y = r\sin\theta$ and simplifying Equation A.6 yields,

$$A' = \int_0^{2\pi} \int_0^{u} \frac{R_{ext}}{\sqrt{R_{ext}^2 - r^2}} r\,dr\,d\theta \tag{A.7}$$

The solution to this integral is,

$$A' = 2\pi R_{ext}(R_{ext} - \sqrt{R_{ext}^2 - u^2}) \tag{A.8}$$

The constant u is dependent on ψ, and is equated as,

$$u = R_{ext}\sin\psi/2 \tag{A.9}$$

The total electropermeabilization surface area of both hemispherical end cap sections is thus $2A'$.

Electropermeabilization of the cylinder is assumed to occur below an effective membrane thickness, T. This thickness is determined by the set electropermeabilization angle, ψ. To obtain a value for T, consider a plan view of the cylinder whose long axis is perpendicular to an applied electric field along the y, as shown in Figure A.3. In this case,

$$x_T = R_{int}\sin\psi/2 \tag{A.10}$$

where R_{int} is the internal cylinder diameter and x_T is the x component of R_{int} at the angle $\psi/2$. The y component of R_{int}, y_{int}, is,

$$y_{int} = \sqrt{R_{int}^2 - x_T^2} \tag{A.11}$$

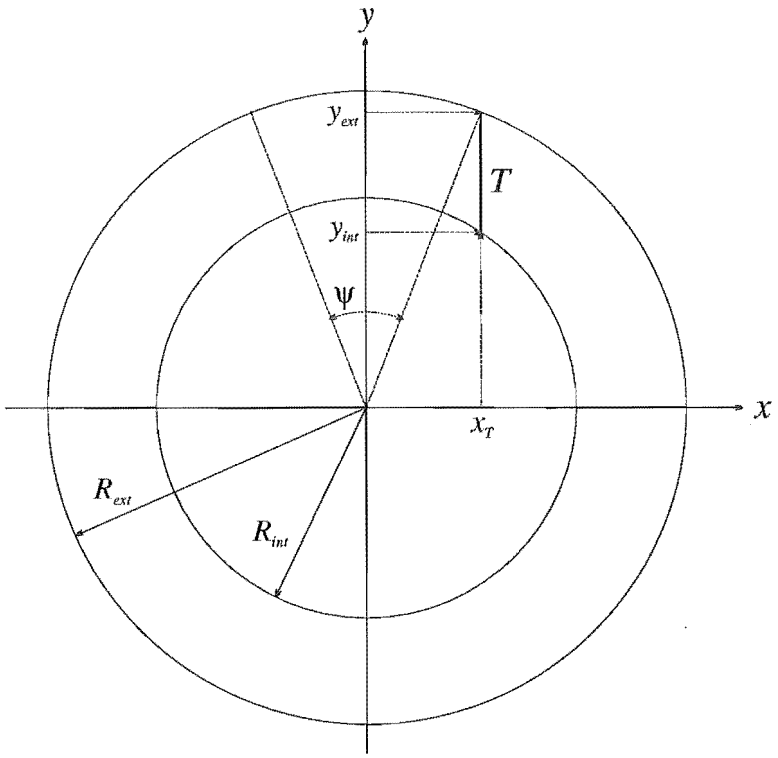


Figure A.3 Plan view of cylinder section showing membrane thickness, T, for an applied electric field in the y direction

and the y component of R_{ext} is,

$$y_{ext} = \sqrt{R_{ext}^2 - x_T^2} \tag{A.12}$$

Therefore, T is defined as,

$$T = \sqrt{R_{ext}^2 - x_T^2} - \sqrt{R_{int}^2 - x_T^2} \tag{A.13}$$

Figure A.4 illustrates the cylinder oriented at some angle, ν, in the x, y plane, from the x axis (the axis of an applied electric field) and its projection through the x, y plane. The equation of this projection is,

$$\frac{x^2}{(R_n/\cos\nu)^2} + \frac{y^2}{R_n^2} = 1 \tag{A.14}$$

where n is either *ext* or *int*. As the cylinder is rotated about the y axis, the x component of the projection will vary with ν. Equation A.14 then becomes,

$$\frac{x_{ext}^2 \cos^2\nu}{R_{ext}^2} + \frac{y^2}{R_{ext}^2} = 1 \tag{A.15}$$

for the outside surface and,

$$\frac{x_{int}^2 \cos^2\nu}{R_{int}^2} + \frac{y^2}{R_{int}^2} = 1 \tag{A.16}$$

for the inside surface. Combining Equation A.15 and Equation A.16 to eliminate y^2 yields,

$$x_{ext}^2 \cos^2\nu + R_{int}^2 - x_{int}^2 \cos^2\nu = R_{ext}^2 \tag{A.17}$$

From Figure A.4, T is equated as,

$$T = x_{ext} - x_{int} \tag{A.18}$$

Rearranging Equation A.18 and squaring produces,

$$x_{int}^2 = T^2 - 2Tx_{ext} + x_{ext}^2 \tag{A.19}$$

Substituting Equation A.19 into Equation A.17 gives,

$$x_{ext}^2 \cos^2\nu + R_{int}^2 - (x_{ext} - 2x_{ext}T + T^2)\cos^2\nu = R_{ext}^2 \tag{A.20}$$

Solving for x_{ext},

$$x_{ext} = \frac{R_{ext}^2 - R_{int}^2}{2T\cos^2\nu} + \frac{T}{2} \tag{A.21}$$

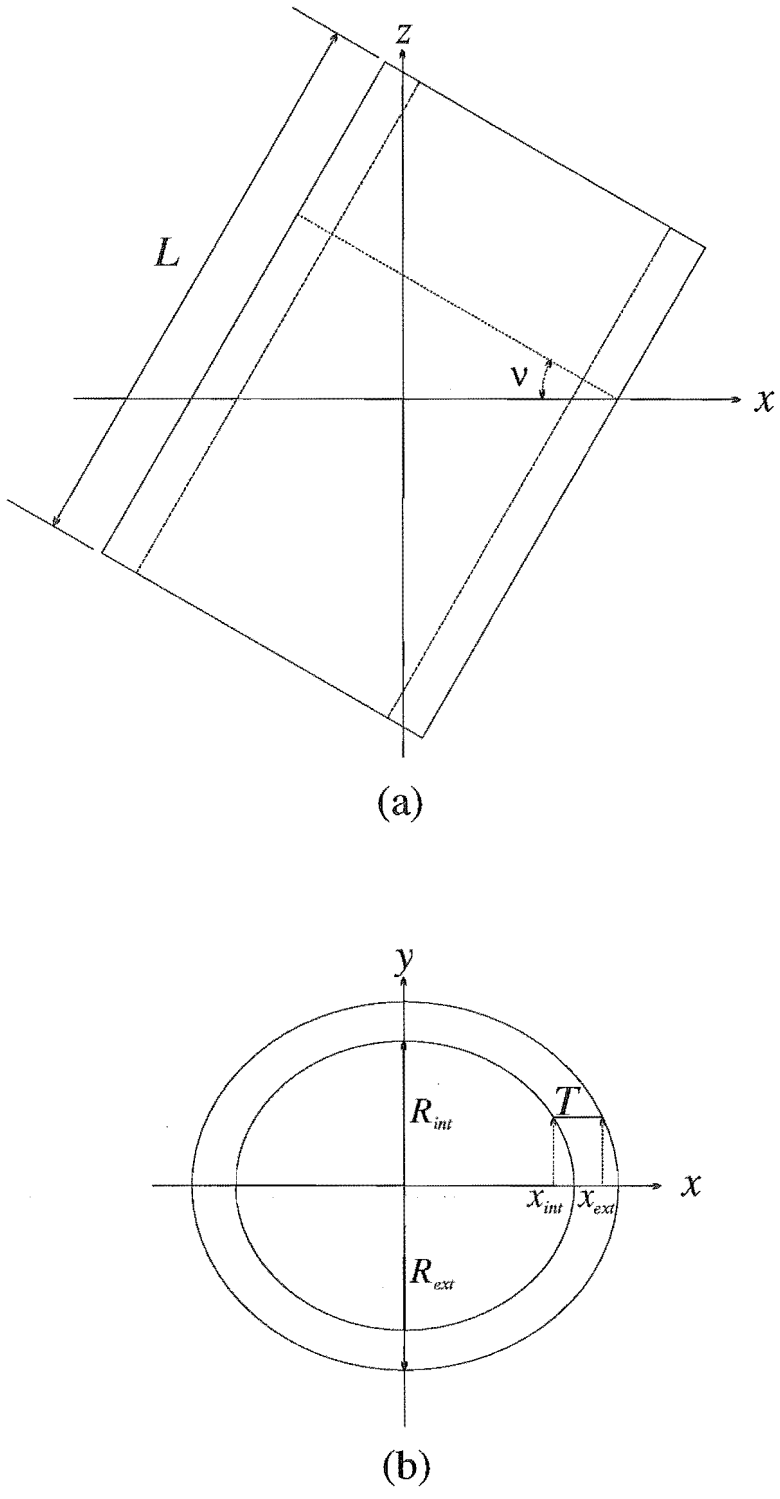


Figure A.4 (a) Cylinder section oriented at an angle ν from the x axis and (b) its projection on the x, y plane

An arc length C is defined on the cylinder oriented perpendicular to its long axis as shown in Figure A.5. The length of C is defined as,

$$C = 2R_{ext}\delta \tag{A.22}$$

where δ is the angle through the origin from the end of C to a line that bisects C.

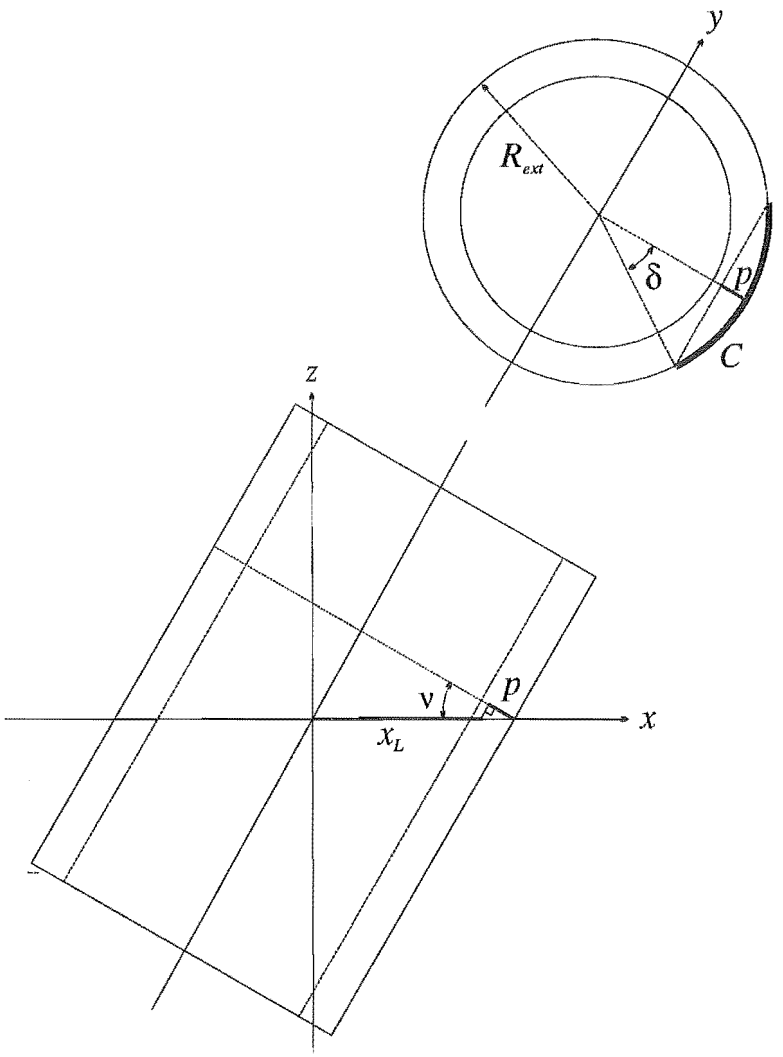


Figure A.5 A curve C defined on the cylinder section

Figure A.5 also shows how the distance, p, relates to x_{ext} and hence T. Now,

$$p = R_{ext} - R_{ext}\cos\delta \tag{A.23}$$

and

$$p = R_{ext} - x_{ext}\cos\nu \tag{A.24}$$

Substituting Equation A.23 into Equation A.24 and solving for δ gives,

$$\delta = \cos^{-1}\left(\frac{x_{ext}\cos\nu}{R_{ext}}\right) \tag{A.25}$$

Thus,

$$C = 2R_{ext}\cos^{-1}\left(\frac{x_{ext}\cos\nu}{R_{ext}}\right) \tag{A.26}$$

The electropermeabilization area of the cylinder, A_{cyl} can then be defined as,

$$A_{cyl} = 2CL \tag{A.27}$$

where L is the length of the cylinder section. In order to determine the average electropermeabilization area for the cylinder in a random orientation, A_{cyl} must be integrated over ν. Due to the symmetry of the cylinder, the limits of the integral can be set from 0 to ν_{crit} where,

$$\nu_{crit} = \cos^{-1}\left(\frac{R_{ext} - R_{int}}{T}\right) \tag{A.28}$$

This is where the x, y plane projection of the cylinder shows T lies on the x axis so that $x_L = R_{ext}$ and $\delta = 0$. The integral, I is thus,

$$I = 1/2\pi \int_0^{\nu_{crit}} A_{cyl}\, d\nu \tag{A.29}$$

The average entire electropermeabilization area, $A_{T.rand}$ for a colloidal suspension of rod shaped cells is,

$$A_{T.rand} = 2A' + I \tag{A.30}$$

If all the cells are aligned with their long axis perpendicular to the applied electric field (i.e. where $\nu = 0$), then the average electropermeabilization area, $A_{T.align}$ is,

$$A_{T.align} = 2A' + 2CL \tag{A.31}$$

If $\psi = \pi/8$, $L = 6R_{ext}$ and $R_{int} = 0.999R_{ext}$, then the ratio of $A_{T.align}$ to $A_{T.rand}$ is about 6:1.

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